(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 April 2001 (05.04.2001)

PCT

(10) International Publication Number
WO 01/23004 A1

(51) International Patent Classification[7]: A61K 48/00, A01N 63/00, C12Q 1/68, C12N 5/00, 15/63

(21) International Application Number: PCT/US00/27212

(22) International Filing Date: 2 October 2000 (02.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/157,224 30 September 1999 (30.09.1999) US

(71) Applicant *(for all designated States except US)*: THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104-3147 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MOLNAR-KIMBER, Katherine [US/US]; 2795 Postshop Road, P.O. Box 190, Worcester, PA 19490 (US). TOYOIZUMI, Takane [JP/JP]; 25-15-401 Yokoyama-cho, Hachiouji-shi, Tokyo 192-0081 (JP).

(74) Agent: MCCONATHY, Evelyn, H.; Dilworth Paxson LLP, 3200 Mellon Bank Center, 1735 Market Street, Philadelphia, PA 19103-7595 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/23004 A1

(54) Title: REPLICATION SELECTIVE ADENOVIRUSES FOR USE IN CANCER THERAPY

(57) Abstract: The invention provides a replication selective adenovirus (Ad) mutant with improved selectively for tumor cells expressing the tumor associated antigen in cancers and malignancies, as well as in proliferative cells, characterizing diseases, such as restenosis,intimal proliferative disease and pulmonary hypertension. The selected Ad vectors are driven by promoters of the tumor associated antigens, or RNA transcripts or genes therefor, substituting for the activity of at least adenovirus E1A promoter, which has been deactivated or diminished. Also provided is the use of the Ad vector to deliver therapeutic compositions to patients, as well as a method for treating cancers, such as CEA positive cancers, or proliferative cell diseases in a patient by administering to the patient an effective amount of the Ad vector, which may also express a therapeutic gene or peptide, and treatment may also be combined with radiation, chemotherapy or immunomodulatory agents. The Ad is designed to replicate within the tumor cell, thereby spreading throughout the tumor nodule. This permits the delivery of a much higher dose of the heterologous therapeutic protein than previously possible, and the virus achieves a direct, oncolytic effect on the tumor.

Replication Selective Adenoviruses for Use in Cancer Therapy

REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application 60/157,224 filed September 30, 1999.

GOVERNMENT SUPPORT

This work was supported in part by grants from the National Institutes of Health, grant numbers RO1-74958, RO1 CA69646 and PO1-CA66726.

FIELD OF THE INVENTION

This invention relates generally to the field of molecular biology, and more specifically to methods for killing, inhibiting or reducing hyperproliferating cells, tumors or malignancies that are causing a disease state or condition, including but not limited to cancers or primary pulmonary hypertension.

BACKGROUND OF THE INVENTION

Cancer remains one of the leading causes of morbidity and mortality in humans worldwide. Although surgery, chemotherapy and radiation have been utilized with some success to cure cancer, novel strategies are warranted to treat the remaining tumors. One of the hurdles to overcome in most forms of somatic gene therapy is the specific delivery of the therapeutic gene (encoding a therapeutic protein) to the organs manifesting the disease. Replication selective viruses, *i.e.*, viruses that have been shown to replicate in tumor cells better than in normal cells, have shown promise as oncolytic agents.

Adenovirus (Ad) is a naturally-occurring large DNA virus whose natural host is human cells, but which display a broad host range. Physically, adenovirus is a medium-sized icosahedral virus containing a double-stranded, linear DNA genome. There are approximately 50 serotypes of human adenovirus, which are divided into six families based on molecular, immunological, and functional criteria. By adulthood, virtually every human has been infected with the more common adenovirus serotypes, the major effect being cold-like symptoms.

The infectious cycle of the adenovirus takes place in 2 steps: the early phase which precedes initiation of the replication of the adenoviral genome, and which permits production of the regulatory proteins and proteins involved in the replication and transcription of the viral DNA, and the late phase which leads to the synthesis of the structural proteins. The early genes are distributed in 4 regions which are dispersed in the adenoviral genome, designated E1 to E4 (E denotes "early"). The early regions comprise at least-six transcription units, each of which possess their own promoters. The expression of the early genes is itself regulated, some genes being expressed before others. Three regions, E1, E2 and E4, respectively, are essential to replication of the virus. Thus, if an adenovirus is defective for one of these functions, that is to say if one or more essential proteins encoded by any one of these regions cannot be produced, this protein will have to be supplied to it in trans, or the virus cannot replicate.

The E1 early region is located at the 5' end of the adenoviral genome, and contains 2 viral transcription units, E1A and E1B, respectively. This region encodes proteins, which participate very early in the viral cycle and are essential to the expression of almost all the other genes of the adenovirus. In particular, the E1A transcription unit codes for a protein, which transactivates the transcription of the other viral genes, inducing transcription from the promoters of the E1B, E2A, E2B, E3, E4 regions and the late genes.

The adenovirus enters the permissive host cell via a specific receptor, and it is then internalized. The viral DNA associated with certain viral proteins needed for the first steps of the replication cycle enters the nucleus of the infected cells, where its transcription is initiated by cellular enzymes. Replication of the adenoviral DNA takes place in the nucleus of the infected cells and does not require cell replication. Assembly of the new viral particles or virions also takes place in the nucleus, after which they are released from the infected cells, and can infect other permissive cells.

A number of adenoviruses have now been well characterized genetically and biochemically. This is the case with human adenovirus type 5 (Ad5), GenBank M73260. Thus, adenoviruses possess advantageous features, making them vectors of choice for the transfer of genes of interest. Many recombinant adenoviruses are described in the literature, most of which are derived from Ad5, and are defective for

the E1 function, precluding their dissemination into the environment and the host organism. Typically, exogenous sequences are integrated in place of all or part of the E1 and/or E3 region.

Thus, a defective or crippled adenovirus construct refers to an adenovirus construct, which has been manipulated, such that it encodes and expresses a desired gene product, but at the same time is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. Such defective adenoviruses can be propagated only, *e.g.*, in a cell line complementing in trans the E1 function, which is essential to viral replication. At present, a complementation line which is most often used, is the embryonic kidney line 293 (Graham *et al., J. Gen. Virol.*, 36:59-72 (1997), which results from the integration in its chromosomes of a fragment of the Ad5 genome comprising, in particular, the 5' end of the viral genome; so that line 293 complements adenoviruses which are defective for the E1 function.

Consequently, defective adenoviral vectors have been developed, from which certain specific regions of the adenoviral genome have been deleted, and which are better suited to the transfer of an exogenous nucleotide sequence *in vivo*, along with newly, characterized complementation lines, which are acceptable from a pharmaceutical standpoint, and which afford the safety features required for the production of products intended for human use. The value of these new vectors is that they display an increased cloning capacity permitting the insertion of one or more large genes of interest, and afford maximal safety of use. These deleterious mutations render these adenoviruses incapable of autonomous replication and of cell transformation without impairing their capacity to transfer and express a gene of interest.

A major characteristic of these replication defective viruses is that the transduction of the majority of tumor cells in a nodule is often difficult to achieve (Sterman et al., *Hum. Gene Ther.* 9:1083-1092 (1998)). Thus, replication selective or replication conditional viruses that are engineered to replicate at least 10 fold better in tumor cells than normal cells are being evaluated as anti-tumor agents (Kirn, In Gene Therapy of Cancer: Translational Approaches from Preclinical Studies to Clinical Implementation (Lattime *et al.*, eds), Academic Press, San Diego, 235-248 (1999); Bischoff *et al.*, *Science* 274:373-376 (1996); Wildner *et al.*, *Cancer Res.* 59:410-413

(1999a); Wildner *et al.*, *Gene Ther.* 6:57-62 (1999b); Coukos *et al.*, *Gene Ther. Mol. Biol.* 3:79-89 (1999b), Toyoizumi *et al.*, *Hum. Gene Ther.* 10: 3013-3029 (1999) and references therein).

The first replication selective Ad for cancer therapy described was Add*l*520 or ONYX-015 (Kirn, 1999); Bischoff *et al.*, 1996). It has a deletion in the E1B p55K protein of Ad. Because of its safety profile in human phase I trials and sufficient evidence of potential efficacy in phase II, ONYX-015 is currently being evaluated in phase III clinical trials for head and neck cancer. The oncolytic activity of the replication selective adenovirus, ONYX-015, can be augmented by chemotherapy, radiation or co-expression of a suicide gene followed by pro-drug administration (Yu *et al.*, *Cancer Res.* 60:1009-1013 (2000); Khuri *et al.*, *Nat Med.* 6:879-885 (2000); Lamont *et al.*, *Ann Surg Oncol.* 7:588-592 (2000); Freytag, *et al.*, *Hum. Gene Ther.* 9:1323-1333 (1998)).

Blaese and collaborators have also engineered recombinant Ad with deletions of the p55K protein and the inclusion of a suicide gene (Wildner *et al.*, 1999a; Wildner *et al.*, 1999b). Although these recombinant Ad have been shown to be oncolytic in some tumor cells, the factors determining tumor sensitivity are complex and not fully characterized (Goodrum *et al.*, *J. Virol.* 72:9479-9490 (1998); Hay *et al.*, *Hum. Gene Ther.* 10:579-590 (1999); Rothmann *et al.*, *J. Virol.* 72:9470-9478 (1998); Turnell *et al.*, *J. Virol.* 73:2074-2083 (1999)). For example, it was originally proposed that these viruses would be useful for tumors with p53 mutations. However, tumors with normal p53 have also been shown to be susceptible. Thus, there is no known protein, set of proteins or RNA that can be used to predict susceptibility of these replication selective viruses.

The ideal replication selective adenoviral mutants would exhibit tumor selectivity that can be predicted by the presence of one or more proteins or RNA molecules. Additionally, the innovative ideal vector would replicate and be oncolytic in dividing as well as nondividing tumor cells, and exhibit a favorable safety profile which will allow systemic administration of vector to target metastatic lesions.

Several strategies for engineering replication selective adenoviruses with more predictable tumor specificity have recently emerged. The insertion of Prostate Specific Antigen (PSA) promoter upstream of the E1A gene at nucleotide 552 was

reported to replicate approximately 20-100-fold higher in PSA positive tumor lines than PSA negative lines (Rodriquez *et al.*, *Cancer Res.* 57:2559-2563 (1997)). Ad764 in which PSA promoter regulated E1A and human glandular kallikrein-2 (hK2) promoter regulated E1B region exhibited approximately 10,000-fold selectivity for prostate cancer cells versus normal cells (Yu *et al.*, *Cancer Res.* 59:1498-1504 (1999)). Although there were earlier reports of Ad in which the promoter of Alpha Feta Protein (AFP) regulated the E1A region, the selectivity for hepatocellular carcinoma cells of replication selective Ad was enhanced in an Ad in which the three modifications were made: (a) AFP regulated E1A, (b) p55K protein was deleted (similar to ONYX-015) and (c) the early promoter of cytomegalovirus (CMV)-regulated expression of E1B p19K (Hallenbeck *et al.*, *Hum. Gene Ther.* 10:1721-1733 (1999)).

Such earlier reported viruses all insert a promoter of a tumor associated gene or tissue associated gene near the translational start site of the E1A genes. However, there is some baseline level of expression of E1A genes that still occurs. Regulation of transcription from the E1A region involves several elements illustrated in Figure 1.

These several transcriptional regulatory elements of the E1A promoter include: ITR (Inverted terminal repeat); 2 type I enhancers, 6 type II enhancers, a CAAT box, a TATA box and a CAP site (transcriptional start site) as shown in Figure 1. The Enh1 (Enhancer elements type I) enhance the transcriptional activity of the E1A promoter. Enh2 (Enhancer elements type II) augment the transcriptional activity of the Ad5 promoters (the early promoters, E1, E2, E3, E4, and the major late promoter (MLP)). A1-7 are packaging sequences, essential for efficient loading of the Ad5 genome into the capsid, and thus for the efficient production of infectious Ad virions. The Ad packaging elements required for efficient production of infectious adenoviral particles have been mapped to overlapping sequences (Hearing *et al.*, *J. Virol.* 61:2555-2558 (1987)).

The TATA box is a common sequence that is involved in transcriptional regulation. Removal of the TATA box and cap site transcriptional regulatory elements has been shown to decrease the promoter activity and the transcription of the E1A promoter. Furthermore, the E1 enhancer type I and type II elements, with concurrent insertion of a second copy of packaging elements in the right hand end of

Ad genome, were shown to augment transcription from the E1A region (Hearing *et al.*, *Cell* 45:229-36 (1986)). A detailed map of the left end of the Ad5 genome is shown in Figure 2A.

Inhibition of the baseline level of expression from the distant E1A promoter elements may augment the control of the inserted heterologous tumor selective promoter on the E1A genes. Several methods which can decrease expression of a specific RNA and its encoded protein are known to those skilled in the art and include, but are not limited to, (a) deletion or mutation of the relevant promoter to decrease its transcriptional activity (Hearing *et al.*, 1986), (b) by expression of an anti-sense mRNA transcript (Cox *et al.*, *Lung Cancer* 27:81-100 (2000)), and (c) by addition of negative regulatory sequences (Ogbourne *et al.*, *Biochem. J.* 331:1-14 (1998)). Any methods, or combination of methods, that diminish the transcriptional activity of the E1A promoter, may improve the specificity of the replication selective adenoviruses. In addition, many laboratories have shown that some genes are expressed mostly in tumors in the adult. These genes have been termed "tumor associated antigens" or "tumor associated genes."

Promoters of tumor-associated antigens / RNA transcripts have been used to direct expression of various transgenes to tumor cells. Tumor selective promoters include, but are not limited to, the promoters of α-fetoprotein (liver) (Kaneko *et al.*, *Cancer Res.* 55:5283-5287 (1995)), carcinoembryonic antigen ("CEA"; stomach/ pancreas/colorectal) (Brand *et al.*, *Gene Ther* 5:1363-1371 (1998); Lan *et al.*, *Gastroenterology* 111:1241-1251 (1996); Ohashi *et al.*, *Jap. J. Cancer Res.* 89:457-462 (1998); Tanaka *et al.*, *Biochem. Biophys. Res. Comm.* 231:775-779 (1997)), prostate-specific antigen ("PSA"; prostate) (Gotoh *et al.*, *J. Urol.*160:220-229 (1998)), Egr-1 (brain) (Manome *et al.*, *Hum. Gene Ther.* 9:1409-1417 (1998)), L-plastin (ovarian) (Chung *et al.*, *Cancer Gene Ther.* 6:99-106 (1999a)), gonatotropin hormone (pituitary) (Lee *et al.*, *J. Clin. Endocrinol. Metabol.* 84:786-794 (1999), glycoprotein hormone α subunit (pituitary) (Lee *et al.*, 1999), hexokinase II (lung) (Katabi *et al.*, *Hum. Gene Ther.* 102:155-164 1995 (1999)), glial fibrillary acidic protein (astrocytoma) (Morelli *et al.*, *J. Gen. Virol.* 80 (Pt30):571-583 (1999), neuronal-specific enolase (brain) (Morelli *et al.*, 1999), MUC-1 ("DF-3"; breast, ovarian)(Chen *et al.*, *J. Clin. Invest.* 96:2775-2782 (1995); Tai *et al.*, *J. Clin. Oncol.*

16:2583-2590 (1998)), tyrosinase (skin) (Siders *et al.*, *Cancer Gene Ther.* 5:281-291 (1998)), and ERBB2 (breast) (Vassaux *et al.*, *Gene Ther.* 6:1192-1197 (1999)), and any promoters which have been shown to selectively express heterologous proteins in a tumor specific manner, such as in vectors based on adenovirus, retroviruses or herpes simplex virus.

Two promoters that are regulated during the cell cycle, E2F-1 (brain) (Parr *et al.*, *Nat. Med.* 3:1145-1149 (1997)), and c-myb (Chung *et al.*, *J Virol.* 73:7556-64 (1999b)) may also be useful for directing the expression of E1A genes of adenovirus. The transcriptional regulatory sequences of proteins or RNA molecules that are expressed predominantly in tumor cells include, but are not limited to, survivin (Liu *et al.*, *Biochem J.* 344:305-311 (1999)), telomerase (TER and TERT), IGFII, and H19 and other promoters of oncofetal genes. In addition, some tissue specific promoters, may also be useful for targeting the replication of replication selective adenoviruses to an appropriate tumor.

Both radiation and chemotherapy have significantly contributed to the treatment of cancer. However, impediments to successful therapy by either form of treatment still remain. For example, some tumor types fail to respond to either radiation or chemotherapy. In other instances, originally responsive malignant cells may experience a relapse and become resistant to treatment. Thus, there remains a need in the art for developing additional cancer treatments, which can improve upon or enhance responses to existing therapies, such as chemotherapy or irradiation.

The feasibility of gene transfer and tumor lysis using adenoviruses has been well established (Kirn, 1999; Bischoff *et al.*, 1996; Wildner *et al.*, 1999a; Wildner *et al.*, 1999b; (Sterman *et al.*, *Hum. Gene Ther.* 9:1083-1092 (1998)). However, the question of their safety has not yet been settled and is being evaluated in clinical trials. For example, it is feasible that a recombination event during the production of clinical batches of the recombinant viruses in 293 cells or PerC6 cells could restore the E1 function and generate a non-defective recombinant adenovirus capable of dissemination into the environment.

There are at least four possible mechanisms by which replication selective adenoviral mutants could be effective therapeutic agents for treatment of malignancy. First, adenoviral mutants could initiate and complete a lytic infection, thereby lysing

the cells. Second, replication selective Ad mutants could express suicide genes, such as thymidine kinase (HSV*tk*) gene or the cytosine deaminase-thymidine kinase fusion protein(Rogulski *et al., Clin. Cancer Res.* 3:2081-2088 (1997)), which will augment the oncolytic activity. For example, expression of the tk gene combined with subsequent administration of ganciclovir has been shown to lyse adjacent cells by the passage of the toxic metabolites via gap junctions and enhancement of inflammation utilizing retroviral, herpetic and adenoviral vectors (Freedman *et al.*, In Gene Therapy of Cancer: Translational Approaches from Preclinical Studies to Clinical Implementation (Lattime *et al.*, eds), Academic Press, San Diego, 155-176 (1999). Third, administration of adenoviral mutants might be combined with either chemotherapy (including any drug therapy), photodynamic treatment or radiation to enhance the oncolytic activity of each agent (Nielsen *et al., Cancer Gene Ther.* 4:835-846 (1997)). Finally, the virus could function as an immunostimulant, or as a vector that expresses immunstimulatory molecules, resulting in the stimulation of specific immune effector cells against tumor antigens. The latter strategy is particularly appealing because it may offer a long-term, durable response through a "vaccine effect."

Thus, until the present invention, there has remained a need in the art for a replication selective adenovirus having improved selectively for tumor cells expressing the relevant tumor associated antigen.

SUMMARY OF THE INVENTION

The present invention provides a replication selective adenoviruses with improved selectively for tumor cells expressing the relevant tumor associated antigen. Specifically, the invention provides replication selective adenoviruses under the control of a tumor specific promoter, which can be utilized for treatment of tumors expressing the relevant tumor antigen(s). Moreover, such replication selective adenoviruses, regulated by tissue specific or disease specific promoters, can be used to treat proliferative diseases, such as restenosis, intimal proliferative disease and primary pulmonary hypertension.

Replication sensitive adenoviruses replicate at a level 10-fold or greater in cancer cells, than they do in normal cells, providing a vector that can effectively be

used for cancer treatment. Replication-defective and deletion variants of adenovirus are known in the prior art to be useful, controllable vectors for delivering exogenous genes in gene therapy to the host cells. The viruses of the present invention can be propagated in cells that are positive for the relevant tumor associated antigen, thereby eliminating the potential of recombination with the E1A sequences in 293 cells or PerC6 cells.

The present invention provides a new and useful replication selective virus, developed by the inventors, in which the promoter of the tumor-associated antigen regulates the E1 region, and these viruses can preferentially reduce tumor growth of tumors that express an analogous tumor associated gene, as exemplified by carcinoembryonic antigen (CEA), at levels of greater than 2 ng CEA/10^6. The CEA/ replication selective adenovirus mutant construct has been evaluated *in vivo* against two different representative types of tumors (A549 non-small lung carcinoma and HELA cervical cancer).

In brief, the inventors have crippled at least the E1A promoter so that currently characterized and future tumor selective promoters can be used for regulation of adenovirus replication. Various methods can be used to decrease the activity of the E1A promoter. The principle can also be extended to the E1B promoter and / or the E4 promoter, enhancing the specificity of the adenovirus for tumor cells, and decreasing replication in normal cells. An additional advantage of this technology over the prior art vectors associated with a replication defective adenovirus backbone, is the ability of the replication selective adenovirus to replicate within the tumor cell, thereby spreading throughout the tumor nodule. This permits the delivery of a much higher dose of the heterologous therapeutic protein than previously possible, and achieves a direct, oncolytic effect on the tumor.

An advantage of this technology over ONYX-015 and replication selective Ad based on deletion of the E1B p55K protein is that the ability of the viruses generated using this technology to replicate in a given tumor specimen can be predicted by the assessment of the expression level of the relevant tumor associated antigen.
The invention further relates to an anti-tumor viral oncolytic agent that is effective against tumor cells of a mammal, bird, fish, or the like. The mammal may, for

example, be a human afflicted with a cancer or tumor, or one suffering from a hyperproliferative disease state.

Thus, the invention provides a replication selective adenovirus mutant under the control of a tumor or tissue specific promoter, wherein said adenovirus selectively replicates at an increased level within a cancer or hyperproliferative cell, as compared with replication in a normal cell, permitting the adenovirus to spread throughout a tumor nodule or hyperproliferative cells, and wherein at least adenovirus E1A promoter has been deactivated or crippled, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type adenovirus.

The invention also provides improved selectivity *in vivo* for any target cell which expresses the above-defined adenovirus vector, particularly for the following tumors or hyperproliferative cells, including, but not limited to cancers, carcinomas, sarcomas or neoplasms of the prostate, lung, gastrointestine, colorectum, pancreas, breast, ovary, cervix, stomach, thyroid, mesothelioma, liver, kidney, skin, head and neck, brain, as well as leukemias and lymphomas.

The invention further provides a method for delivering to a target cell a heterologous gene or gene fragment encoding a therapeutic peptide or polypeptide, by delivering the above-defined adenovirus vector, wherein said vector is under the control of a tumor specific promoter, and wherein at least adenovirus E1A promoter is disabled or crippled.

Also provided is a method of treating a patient suffering from a cancer, carcinoma, sarcoma, neoplasm, leukemia, lymphoma or hyperproliferative cell disease by administering to the patient a therapeutically effective amount of a replication selective adenovirus mutant, which is under the control of a tumor specific promoter, wherein said adenovirus selectively replicates at an increased level within the cancer, sarcoma, neoplasm, leukemia, lymphoma or hyperproliferative cell, as compared with replication in a normal cell, permitting the adenovirus to spread throughout a tumor nodule or hyperproliferative cells, and wherein at least adenovirus E1A promoter has been deactivated or crippled, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type adenovirus. In a preferred embodiment replication selective adenovirus mutant delivers to a target cell

a heterologous gene or gene fragment encoding a therapeutic peptide or polypeptide, or a suicide gene.

In each case, the tumor specific promoter is selected from tumor types that express a tumor associated antigen, and the resulting adenovirus vector has improved selectively for tumors or hyperprolifer-ative cells expressing the tumor associated antigen. Suitable tumor associated antigens, include carcinoembryonic antigen, probasin, prostate specific antigen, prostate specific membrane antigen, erb1 or erb2, α-fetoprotein, Egr-1, L-plastin, gonatotropin hormone, glycoprotein hormone α subunit, hexokinase II, glial fibrillary acidic protein, neuronal-specific enolase, MUC-1 ("DF-3"), tyrosinase, ERBB2, survivin, catalytic subunit of telomerase, IGFII P3 and P4, H19, cell cycle associated proteins, E2F-1, c-myb and the tissue specific promoter, thyroglobulin. In a preferred embodiment the tumor associated antigen is carcinoembryonic antigen.

Also provided is a method for producing an infectious, replication selective adenovirus particle comprising (a) selecting a tumor specific promoter selected from a tumor that express a tumor associated antigen, which is active in a eukaryotic cell; (b) deactivating or crippling at least the adenovirus E1A promoter in a replication selective adenovirus, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type replication selective adenovirus; (c) introducing the promoter of step (a) with the crippled replication selective adenovirus of step (b), thereby placing the replication selective adenovirus under the control of the tumor associated antigen promoter; (d) culturing the adenovirus construct under conditions permitting the uptake of the vector by, and replication in, a host cell expressing the tumor associated antigen; and (e) harvesting the thus produced replication selective adenovirus particle, which is selectably reproduced only in cells expressing the tumor associated antigen. Also provided is the adenovirus particle produced by the preceding method. Further provided is a pharmaceutical composition comprising the subject replication selective adenovirus particle, and a pharmaceutically acceptable carrier therefor, permitting administration of a therapeutically effective amount of the subject adenovirus to the hyperproliferative or cancer target cell of a patient, thereby achieving an oncolytic effect on the target.

The invention further provides a method of inactivating a tumor or hyperprolifer-ative target cell in a patient, comprising steps of (a) through (c) of the preceding method to produce the vector, into which a heterologous gene or gene fragment is introduced to encode a therapeutic peptide or polypeptide, such that it will be expressed from the vector within the target cell; then introducing the loaded vector into the target cell of the patient in a therapeutically effective amount. In preferred embodiments of the invention, this therapeutic method is combined, before treatment, after treatment or concurrently with treatment with chemotherapy, immunostimulation, phototherapy, irradiation, or any combination thereof. In fact, by using the method of the present invention, the patient may be particularly sensitized to such additional therapies.

Additional objects, advantages and novel features of the invention will be set forth in part in the description, examples and figures which follow, and in part will become apparent to those skilled in the art on examination of the following, or may be learned by practice of the invention.

DESCRIPTION OF THE DRAWINGS

The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings, certain embodiment(s) which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities shown.

Figure 1 graphically depicts the left end of Ad5, comprising the following elements: Enh1 (Enhancer elements type I); Enh2 (Enhancer elements type II); transcriptional start site (CAP site, +1); TATA box; and translational start site (ATG, +60). A1-7 are Packaging elements.

Figures 2A-2C graphically depict construction of recombinant replication selective Ad5. Figure 2A is a map of the left end of Ad5, showing the inverted terminal repeat (ITR); enhancer packaging elements; TATA box, transcriptional start site (CAP site, +1), translational start site (ATG, +60). Figure 2B depicts and compares the Ad5 sequences of the two plasmids,

Ad460CEA and Ad522CEA with site of Ad5 deletion and/or insertion of the CEA promoter. In the plasmid, Ad460CEA, the CEA promoter replaced the Ad5 nucleotides 460-522. However in Ad522CEA, the CEA promoter was inserted in between nucleotides 522 and 523. Figure 2C depicts the linearized plasmid and large right end fragment of AdGFP-E3BFP.

Figures 3A-3H depict the cytopathic effects of the replication defective, E1-deleted Add*l*312 (Figures 3A and 3E); wildtype (wt) Ad5 (Figures 3B and 3F); and Ad522CEA (Figures 3C and 3G); and Ad460CEA (Figures 3D and 3H)on the two colorectal carcinoma cell lines, Colo320 and LoVo. Figures 3A through 3D show Colo320 cells infected at MOI of 100. Figures 3E through 3H show LoVo cells infected at a MOI of 0.001. Cells were photographed with a camera at magnification x20, using phase contrast.

Figure 4 depicts the expression levels of CEA in A549 (lane 1), MCF7 (lane 2) and Hela cells (lane 3). Molecular weight standards are shown at right of gel.

Figure 5 graphically depicts the efficacy of Ad460CEA and Ad522CEA on A549 and Hela tumors, as compared with treatment with media alone (control) or wtAd5 (Ad.E3tk). Each bar represents the mean $\pm$ S.E. of at least 2 experiments. *$p \leq 0.05$. **$p \leq 0.01$.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides replication selective adenoviruses with improved specificity for tumors, wherein the activity of the naturally occurring adenovirus E1A promoter is crippled, deleted or decreased, and a second promoter is added to regulate E1A expression, wherein the second promoter is selected from a gene whose expression is restricted or strongly associated with a cancer ("tumor associated antigen" or RNA). As exemplified, when an adenovirus construct was formed in which the promoter of the carcinoembryonic antigen (CEA) was used to replace the TATA box and transcriptional start site of the adenovirus E1A promoter, a replication selective adenovirus was generated by homologous recombination in 293 cells (see Ad460CEA in comparison to Ad522CEA in Figure 2B). The promoter of the tumor associated antigen or RNA regulates the E1 region and these viruses can

preferentially reduce growth of tumors that express the analogous tumor associated gene.

As an initial prototype of the present invention, the inventors have engineered the well-characterized promoter of carcinoembryonic antigen (CEA) to regulate the expression of the E1A genes of Adenovirus 5. Carcinoembryonic antigen (CEA) is a widely used marker observed in several tumors, including colorectal carcinoma, gastric carcinoma, and pancreatic cancer, lung, breast, cervical cancer, and ovarian cancer (Kim *et al., Int J Cancer* 52:718-725 (1992); Shinkai *et al., Cancer* 57:1318-1323 (1986); Ngan *et al., Tumour Biol*;19:439-444 (1998)).

The CEA promoter was selected because it has been fully mapped and CEA expression is prevalent in many cancers. The promoter elements critical for selective expression of transgenes in recombinant adenoviruses are located between nucleotide -299 to nucleotide +111 (the translational start site)(Richards *et al., Hum. Gene Ther.* 6:881-893 (1995)). Multiple tandem repeats of the CEA enhancer can augment the specificity of CEA promoter (Richards *et al.*, 1995; Schrewe *et al., Mol Cell Biol.* 10:2738-2748 (1990)). However, similar results may be obtained with other promoters, and the invention is not intended to be limited to a promoter from a particular tumor associated antigen.

There are at least four possible mechanisms by which replication selective adenoviral mutants can be effective therapeutic agents for treatment of malignancy. First, adenoviral mutants can initiate and complete a lytic infection, thereby lysing the cells. Second, replication selective Ad mutants can express suicide genes such as thymidine kinase (HSV*tk*) gene or the cytosine deaminase-thymidine kinase fusion protein, which will augment the oncolytic activity. Third, administration of adenoviral mutants may be combined with either chemotherapy, photodynamic or radiation to enhance the oncolytic activity of each agent. Fourth, the virus may also be useful in cancer therapy by acting as an immunostimulant or as a vector for expression of immunstimulatory molecules such as IL-12, resulting in the stimulation of specific immune effector cells against the tumor antigens. This latter strategy is particularly appealing, because it may offer a long-term, durable response through a "vaccine effect."

The replication selective adenoviruses can be engineered by a variety of methods utilizing techniques known in the art, and are not limited to one particular method. These include the following:

(1) by replacing the TATA box and transcriptional start site of the E1A promoter with a tumor selective promoter;

(2) by mutating the two E1A type I enhancers as well as replacing the TATA box and transcriptional start site of the E1A promoter with a tumor selective promoter;

(3) by mutating the two E1A type I enhancers, replacing the TATA box and transcriptional start site of the E1A promoter with a tumor selective promoter, and replacing the E1B promoter with the same or distinct tumor selective promoter;

(4) by mutating the two E1A type I enhancers, replacing the TATA box and transcriptional start site of the E1A promoter with a tumor selective promoter and inserting a promoter which is highly expressed in normal cells but not expressed in tumor cells downstream of the E1A region to result in the transcription of an anti-sense transcript of E1A region in normal cells, thus enhancing the specificity for tumor cells;

(5) by mutating the two E1A type I enhancers, replacing the TATA box and transcriptional start site of the E1A promoter with a tumor selective promoter, and inserting a promoter which is highly expressed in normal cells but not expressed in tumor cells downstream of the E1A region to result in the transcription of an anti-sense transcript of E1A region in normal cells, thus enhancing the specificity for tumor cells. In addition, the E1B promoter is replaced with the same or distinct tumor selective promoter;

(6) by mutating the two E1A type I enhancers, replacing the TATA box and transcriptional start site of the E1A promoter with a tumor selective promoter, and inserting a promoter which is highly expressed in normal cells but not expressed in tumor cells downstream of the E1A region to result in the transcription of an anti-sense transcript of E1A region in normal cells, thus enhancing the specificity for normal cells. In addition, the E1B promoter left intact but the same or additional tumor selective promoter is inserted in between the E1B promoter and regulates the expression of E1B region to a significant extent.

(7) ;by using additional combinations of strategies for crippling E1A promoter coupled with insertion of a tumor selective promoter;

(8) by using additional combinations of strategies for crippling E1A promoter coupled with insertion of a tumor selective promoter and by regulation of E1B or E4 genes with same or distinct tumor selective promoters in which the promoters of E1B or E4 were or were not crippled;

(9) any of the above mentioned viruses can also be engineered to express a suicide gene(s), a tracking gene(s), a gene(s) to enhance the induction of an immune response to the tumor, an anti-angiogenic gene, and / or a gene that triggers apoptosis. For example, herpes simplex virus type I thymidine kinase, cytosine deaminase, beta galactosidase, B7.1, GM-CSF, TNFalpha, endostatin, IL-12 can be suitable transgenes.

The viruses of the present invention can be used to treat tumors that express the corresponding tumor associated antigen and/or RNA transcript or tissue specific gene therefor. In the case of the Ad460CEA virus, it can be used to treat CEA positive cancers, which include, but are not limited to many gastrointestinal cancers such as pancreatic, gastric, colorectal carcinoma, patients with CEA positive ovarian cancer, patients with CEA positive non small cell lung cancer or breast cancer. The administration of these replication selective adenoviruses may be combined with treatment by radiation, chemotherapy, immunomodulatory agents to enhance the influx and activation of antigen presenting cells and T cells and the induction of an immune response to the cancer or tumor.

Any modification that decreases or prevents the activity of the E1A promoter, such as deletions, mutations and /or insertions into the E1A promoter or type I enhancers would be considered to be within the intent of the present invention. Any modification that decreases or prevents the activity of the E1B promoter, such as deletions, mutations or insertions into the E1B promoter or a combination of the modifications would be considered to be within the intent of the present invention.

As used herein, the phrase "gene therapy" refers to the transfer of genetic material (*e.g.*, DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition. The genetic material of interest encodes a product (*e.g.*, a protein polypeptide, peptide or functional RNA) whose production *in vivo* is

desired. For example, the genetic material of interest can encode a hormone, receptor, enzyme or (poly) peptide of therapeutic value. Examples of genetic material of interest, include but are not limited to DNA encoding: suicide genes, anti-angiogenic molecules, immunostimulatory molecules, tracking genes, proteins of involved in cell death including the adenovirus death protein.

One major obstacle to be overcome before gene therapy becomes a viable treatment, is the development of appropriate vectors to infect tissue manifesting the disease and deliver the therapeutic gene. Since viruses have evolved very efficient means to introduce their nucleic acid into cells, many approaches to gene therapy make use of engineered defective viruses. However, the use of viruses *in vivo* raises safety concerns. Although potentially safer, the use of simple DNA plasmid constructs containing minimal additional DNA, on the other hand, is often very inefficient and can result in transient protein expression.

As used herein, the term "introduce" or "introducing" in relation to nucleic acid encompasses any method of inserting a heterologous or exogenous nucleic acid molecule into a cell and includes, but is not limited to transduction, transfection, microinjection and viral infection of host cells. The terms "heterologous" and "exogenous" may be used interchangeably, and refer to any DNA sequence not found in the native vector genome. Methods of carrying out these procedures are well known to those of skill in the art. The nucleic acid may be introduced, for example, by contacting the cell with an adenovirus vector, a retroviral vector, a herpes virus vector, or the like.

In a preferred embodiment, the nucleic acid is introduced into the cell by contacting a cell with a replication selective adenovirus vector containing heterologous nucleic acid under conditions such that the nucleic acid is inserted into the cell. In the most preferred embodiment of this invention, the virus is a replication-incompetent adenovirus or retrovirus.

In the present invention, the terms "replication-conditional," "replication selective," and "crippled" are used essentially interchangeably. For the purposes of the present invention, the term "deletion" or "lacking" refers to the elimination of at least one nucleotide in the target region, and the deletion can naturally be continuous or discontinuous. All or part is taken to mean either the whole or only a portion of the

region in question. Deletions are preferred which disrupt the activity of the promoter and/or enhancers. Hence, they may lie in a regulatory region such as the promoter region, and may affect at least one nucleotide so as to destroy or render a promoter region non-functional.

The deletions in question may also comprise partial deletions of one or more genes of said region or of the whole of the region.

A replication-selective adenovirus is defined as a virus that replicates at least 3-10 fold better in cells expressing the relevant tumor associated antigen than in normal cells, precluding spread of the vector in the infected host cells. Examples of such vectors useful for the practice of this invention are well known and readily available to those of skill in the art.

A "cloning vehicle," "expression vehicle," or "vector" refers to a plasmid, phage DNA, cosmid, virus, or other DNA sequence, such as an adenovirus, in particular a replication selective adenovirus, which is able to replicate in a host cell, characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without attendant loss of an essential biological function of the DNA, *e.g.*, replication, production of coat proteins or loss of promoter or binding sites, and which contain a marker suitable for use in the identification of transformed cells, *e.g.*, antibiotic resistance, such as tetracycline resistance or ampicillin resistance. In general, "cloning" refers to a process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

The expression control sequence employed in this invention, is determined by its activity in the "target" cells of interest (tumor cells or host cells) and a variety of factors, *e.g.*, number of sites susceptible to a particular restriction enzyme, activity of the promoter in target cells in comparison to normal cells, and other factors recognized by those of skill in the art. The choice of a site in the adenovirus vector, the expression control sequence, and strategy for decreasing the activity of the E1A/ E1B and/ or E4 promoter(s) is determined by a balance of these factors, not all selections being equally effective for a given case.

A "gene" is defined as any nucleic acid sequence that encodes an active or functional molecule. As used herein, the term "nucleic acid" means DNA, including

cDNA, or RNA. A "cDNA clone" refers to a clone containing a DNA insert that was synthesized from mRNA and does not contain introns. The gene may be an oncogene, a tumor suppressor gene, it may encode therapeutic molecules including antisense or ribozyme RNAs, a gene encoding an enzyme, a gene encoding a cytokine or other immune modulating macromolecule, a gene encoding a recombinant antibody, a gene encoding a lytic peptide, a gene encoding a vaccine antigen, a gene encoding a macromolecule which complements genetic defects in somatic cells, or a gene encoding a macromolecule which catalyses processes leading to cell death.

A "recombinant" DNA molecule or a "hybrid" DNA refers to a molecule consisting of segments of DNA from different genomes, which have been joined end-to-end outside of living cells, and which can be maintained in living cells.

Isolated nucleic acids useful in this invention are those that contain substantially no additional nucleic acids, and encode a polypeptide functionally equivalent to a polypeptide encoded by the E1A region of the adenovirus genome. In one aspect of this invention, the isolated nucleic acid is the promoter of the adenovirus E1A region. This region is defined by those of skill in the art to be from nucleotide 560 to nucleotide 1542.

A "genomic clone" refers to a clone containing a DNA insert, which is a fragment of a genome (*i.e.*, isolated from total cellular DNA). It can contain introns, which interrupt
the protein coding region of the gene. By comparison "exon" are those portions of the gene which after transcription are maintained in the mRNA following splicing of the precursor RNA.

A "plasmid" is a nonchromosomal double-stranded DNA sequence comprising an intact "replicon" such that the plasmid is replicated in a host cell. When the plasmid is placed within a unicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for tetracycline resistance (TET^R) transforms a cell previously sensitive to tetracycline into one which is
resistant to it. A cell transformed by a plasmid is called a "transformant."

"Transcription" refers to the process of producing mRNA from a gene or DNA sequence. "Translation" refers to the process of producing a polypeptide from

mRNA. "Expression" refers to the process undergone by a gene or DNA sequence to produce a polypeptide, meaning a combination of transcription and translation.

"Expression control sequences" are those nucleotide sequences that control and regulate expression of genes when operatively linked to those genes. They include promoters of the present invention, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells, and their viruses, or combinations thereof. Both positive and negative regulatory elements are involved in regulation of the genes.

Various expression control sequences may also be chosen to effect the expression of the DNA sequences of this invention. These expression control sequences include those listed above and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses and various combinations thereof.

For expression of the DNA sequences of this invention, these DNA sequences are operatively-linked to one or more of the above-described expression control sequences in the expression vector. Such operative linking, which may be effected before or after the chosen polypeptide DNA sequence is inserted into a cloning vehicle, enables the expression control sequences to control and promote the expression of the DNA sequence.

As used herein the term "functionally equivalent nucleotide sequence" is intended to cover minor variations in the viral vector sequence which, due to degeneracy in the DNA code, does not result in a virus having substantially different biological activities from the native virus, such as an adenovirus. The encoded virus proteins can have an amino acid sequence, which is at least in part different from the native virus sequences, but which should retain substantially the same biological activities as the native viral proteins. This may be achieved by various changes in the sequence, such as insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the virus.

In a preferred embodiment of the present invention, the viral vector includes a cell-specific promoter expressively linked to the gene, such that the gene is expressed only in a desired target cell. The term "promoter" is used according to its art-recognized meaning. It is intended to mean the DNA region, usually upstream to the

coding sequence of a gene or operon, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site. Such promoters are well known in the art. For example, for the treatment of prostate cancer a promoter could be selected from the group including, but not limited to promoters derived from the probasin, prostate specific antigen (PSA) or prostate specific membrane antigen (PSMA) genes. Similarly, for breast cancers, the erbB-2 promoter may be useful. For other cancers such as lung and gastrointestinal carcinoma, the carcinoembryonic antigen (CEA) promoter is preferred. However, the selected promoter need not be limited to particular cancers, so long as the target cell is capable of expressing the promoter-controlled virus vector, bearing a selected heterologous gene.

The CEA promoter can be used to target expression of the heterologous gene transported by the adenovirus vector to cancer cells, as described herein. Carcinoembryonic antigen, or CEA, is a heavily glycosylated, 180,000 dalton protein found at high levels in serum in association with certain cancers, especially those of the gastrointestinal tract, such as colon carcinomas, including a large number of primary and metastatic colorectal tumors. CEA is one of the most widely studied of the oncofetal tumor-associated antigens.

CEA promoters are preferably used to direct the expression heterologous genes in specific cancer cells that express CEA proteins. This refers to any cancer cells that are active in the synthesis of the CEA proteins. Examples of target cancer cells include, but are not limited to, colorectal carcinoma, lung adenocarcinoma, pancreatic carcinoma or other gastrointestinal tumors, and ovarian tumors.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements

are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 base pair apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. By employing a promoter with well-known properties, the level and pattern of expression of a polynucleotide following transfection can be optimized.

Yet another system that could be used according to the present invention utilizes a synthetic promoter. In this system a vector with a promoter is operably linked to a gene whose expression is to be regulated. The promoter could consist of a DNA binding domain, an activation domain, and a ligand binding domain, operably linked together. At least one of these components would not naturally be found in humans.

"Enhancers" were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination could also be used to drive expression of E1A, and E1B and /or E4 regions of adenovirus, and thus its replication.

The ability to transfer a gene into a cell requires a method of transferring the new genetic material across the plasma membrane of the cell, and subsequent expression of the gene product to produce an effect on the cell. There are several

means to transfer genetic material into a cell, including direct injection, lipofection, transfection of a plasmid, or transduction by a viral vector. The natural ability of viruses to infect a cell and direct gene expression make viral vectors attractive as gene transfer vectors.

The term "replication" means duplication of a vector. This duplication, in the case of viruses, can occur at the level of nucleic acid, or at the level of infectious viral particle. In the case of DNA viruses, replication at the nucleic acid level is DNA replication. The essential feature is nucleic acid copies of the original viral vector and the formation of infectious DNA viral particles. Such particles may successively infect cells in a given target tissue, thus distributing the vector through all or a significant portion of the target tissue.

In certain embodiments of the present invention, the viral vectors described herein are used in cells *in vitro* for producing foreign proteins. In addition, the viral vectors may be used in gene therapeutic contexts to provide to therapeutic genes to cells in *ex vivo* and *in vivo* contexts. Finally, it is contemplated that viral vectors of the present invention will find use as attenuated live virus vaccine vectors. It is envisioned that a vector made according to the present invention will provide efficient, and high level expression of a transgene when introduced into a host cell.

The selection of an appropriate cloning or expression host is controlled by a number of factors recognized by the art. These include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination or binding of the protein to be expressed by host cell proteins difficult to remove during purification, ease of recovery of the desired protein, expression characteristics, bio-safety and cost. A balance of these factors must be struck with the understanding that not all host vector combinations may be equally effective for either the cloning or expression of a particular recombinant DNA molecule.

In certain embodiments, the present invention further involves a method for expressing a gene in a mammalian cell. Such methods involve the use of a vector construct containing heterologous DNA encoding the therapeutic gene and a means for its expression, replicating the vector in an appropriate helper cell, obtaining viral particles produced therefrom, and infecting mammalian cells with the recombinant

virus particles. The gene could simply encode a protein for which large quantities of the protein are desired, *i.e.*, large scale *in vitro* production methods. Alternatively, the gene could be a therapeutic gene, for example to treat cancer cells, to express immunomodulatory genes to fight viral infections, or to replace a gene's function as a result of a genetic defect.

In the context of the gene therapy vector, the gene will be a heterologous DNA, and is meant to include DNA derived from a source other than the viral genome, which provides the backbone of the vector. Finally, the virus may act as a live viral vaccine and express an antigen of interest for the production of antibodies. The gene may be derived from a prokaryotic or eukaryotic source, such as a bacterium, virus, yeast, plant, or even an animal. The heterologous DNA may also be derived from more than one source, *i.e.*, a multi-gene construct or a fusion protein. The heterologous DNA may also include a regulatory sequence may be derived from one source and the gene from a different source.

A tumor is a transformed cell, such as a cancer cell, which may express tumor-specific antigens that can serve as targets according to methods and treatments of the invention. Cell death of the tumor or tumor cells may occur directly as a result of gene expression, or indirectly as a result of an immune response to an expressed foreign macromolecule. Preferably the gene encodes an enzyme, such as herpesvirus thymidine kinase or non-mammalian cytosine deaminase, which metabolises a pro-drug, which may be introduced throughout the tumor as the heterologous gene sequence carried by the subject adenovirus. In one embodiment of the present invention, the presence of the appropriate pro-drug, expression of the gene by the infected cell and metabolism of the pro-drug results in a toxic product which leads to cell death.

The promoters selected for use in a preferred embodient of the present invention comprise "tumor selective promoters" or "tumor associated promoters," such as but not limited to CEA(Brand *et al.*, 1998; Lan *et al.*, 1996; Ohashi *et al.*, 1998; Tanaka *et al.*, 1997); survivin (Alteiri *et al.*), telomerase (TER and TERT), IGFII, H19 and other promoters of oncofetal genes, E2F promoter promoters (Parr *et al.*, 1997), MUC1(Chen *et al.*, 1995), and any promoters which have been shown to selectively express heterologous proteins in a tumor specific manner, such as in

vectors based on adenovirus, retroviruses or herpes simplex virus. Additional examples, are set forth in Table 1.

Table 1: Selective expression of transgenes by tumor specific promoters/enhancers in adenoviral cancer gene therapy

Promoter/ enhancer	Malignancy	Transgene	Reference
AFP	Liver Liver Liver Liver Liver	TK TK LacZ CD IL-2	Kaneko et al., 1995 Wills et al., 1995 Arbuthnot et al., 1996 Kanai et al., 1997 Bui, et al., 1997
CEA	Stomach Stomach Pancreas Colon	CD TK TK TK	Lan et al., 1996 Tanaka et al., 1997 Ohashi et al., 1998 Brand et al., 1998
PSA	Prostate Prostate	TK TK, PNP	Gotoh et al., 1998 Martiniello-Wilks et al., 1998
OC	Osteosarcoma Osteosarcoma Osteosarcoma	TK TK TK	Ko et al., 1996 Cheon et al., 1997 Shirakawa et al., 1998
E2F-1	Brain (astrocytoma)	lacZ	Parr et al., 1997
Egr-1	Brain (glioma)	lacZ	Manome et al., 1998
L-plastin	Ovary	lacZ	Chung et al., 1999
GH	Pituitary	LacZ, TK	Lee et al., 1999
Glycoprotein hormone Alpha subunit	Pituitary	LacZ, TK	Lee et al., 1999
HK II	Lung (NSCLC), Breast, Liver	LacZ, TK	Katabi et al., 1999
GFAP	Brain (astrocytoma)	FasL	Morelli et al., 1999
NSE	Brain (astrocytoma)	FasL	Morelli et al., 1999
DF-3 (MUC-1)	Breast	LacZ, TK	Chen et al., 1995
Tyrosine	Melanoma	TK	Siders et al., 1998
ERBB2	Breast, Pancreas	TK	Vassaux et al., 1999

replication defective adenovirus backbone. Although these viruses can be very effective if transduced into many cells, the efficient transduction many cell layers deep within a tumor nodule, or far from an injection site, has been difficult. By comparison, the viruses of the present invention replicate within the targeted tumor cells, expressing the relevant tumor associated antigen carried by the vector, preferrably the adenovirus vector described herein. Thus, it is spread throughout the tumor nodule, delivering a much greater dose of heterologous protein and also exhibiting direct oncolytic effect on the tumor.

The first group of replication selective adenoviruses were based on the deletion of E1B p55K protein (Heise *et al., Nat. Med.* 3: 639-645 (1997a); Heise *et al., Cancer Gene Ther.* (1997b);Heise *et al., Cancer Res.* 59: 2623-2628 (1999)). The first replication selective Ad for cancer therapy described was Add*l*520 or ONYX-015. It has shown efficacy in animal models of various tumors including cervical, ovarian, head and neck cancer. Moreover, the oncolytic activity of ONYX-015 can be augmented by chemotherapy, radiation or co-expression of a suicide gene followed by prodrug administration. The efficacy of this oncolytic adenovirus is now being investigated in phase III clinical trials for treatment of head and neck cancer.

Blaese and collaborators have also engineered recombinant Ad with deletions of the p55K protein and the inclusion of a suicide gene(Wildner *et al.*, 1999a, 1999b). Although these recombinant Ad have been shown to be oncolytic in some tumor cells, the factors determining tumor sensitivity are complex and remain not fully characterized. Certain differences between the previously known replication selective adenoviruses and the ones identified in the present invention are as follows:

(1) These viruses have the E1B p55K protein deleted, whereas Ad460CEA and new derivatives contain the p55K protein;

(2) The specificity of ONYX015 and related viruses for a given patient's sample has to be tested empirically whereas the oncolytic activity of Ad460CEA and the related viruses can be easily predicted by the presence of the expression of the tumor associated protein and the adenovirus receptor (CAR).

Thus, the AD460CEA adenoviruses and related viruses are advantageous over the prior art. The present invention differs from the earlier reported viruses, in that

the present method cripples the endogenous E1A promoter. This advantageously allows the use of currently characterized and future tumor selective promoters for the regulation of adenovirus replication. Secondly, it cripples the E1B promoter, which advantageously allows the use of currently characterized and future tumor selective promoters for regulation of adenovirus E1B expression. The utility of a promoter from a normal cell downstream from the E1A region, which regulates an anti-sense transcript, also enhances the specificity of the virus for tumor cells, and decreases any replication in normal cells. This is particularly advantageous in that it increases specificity of the virus.

According to the present invention, an adenoviral vector according to the invention is derived from the genome of a natural or wild-type adenovirus by deletion or mutation of all or part of the E1A promoter and all or a portion of the E1B promoter region and / or all or a portion of the E4 promoter region comprising the whole of the sequences regulating the expression for the essential early proteins. According to a preferred embodiment, the deletion(s) and/or mutation(s) affect the promoter controlling expression of the E1A region, that is to say the promoter/ enhancer for early proteins, but it does not include all or part of the transcription termination signal which overlaps the sequences coding for the late proteins.

It is an object of these modifications is to reduce or eliminate activity of the transcriptional regulatory sequences of the E1A region, or of the E1B and/ or E4 regions, and change the regulation to that of a promoter for a desired tumor specific antigen or RNA or tissue specific gene and thereby target the replication of the adenoviruses to the target cells.

It is intended that an adenoviral vector according to the present invention can contain one or more of possible modifications of the type identified above, or two or more of them in any combination, or alternatively all possible modifications.

It should be noted that other restriction sites or elements may also be used in conjunction with the deletion of the promoter region to accomplish or enhance the same goal: *i.e.*, diminish the activity of the E1 type I enhancers without interfering with the packaging elements. Secondly, different restriction sites or cre-lox site can be used to insert a tumor selective promoter for regulation of E1B genes.

An adenoviral vector according to the present invention is derived from the genome of a natural or wild-type adenovirus, advantageously a eukaryote, or a human adenovirus, most preferably a human adenovirus type 2, 3, 4, 5 or 7 and, as an absolute preference, a human adenovirus type 5 (Ad5). The preparations are preferably administered mammals (including humans), fish, and avians; more preferably to livestock (including cattle, horses, swine, sheep, goats, etc.), household pets (cats, dogs, canaries, parakeets, etc.) laboratory animals (*e.g.*, mice, rats, rabbits, other rodents, primates, etc.), fish (especially in an aquarium or aquaculture environment, *e.g.*, tropical fish, goldfish and other ornamental carp, catfish, trout, salmon, etc.) and avians, especially poultry such as chickens, ducks, geese, etc.; and most preferably to humans.

This approach provides a means of administering recombinant proteins to these animals for general therapeutic purposes. The uses for animals include the production of immunity (*e.g.*, vaccination) against infectious agents. Also included would be proteins to treat disorders of animals, fish, etc. This strategy may also be used to deliver genes to animals to use the animal to produce proteins for humans use *e.g.*, after purification from biologic fluids of the animal.

When the human Ad5 is used, the deletions of the adenoviral vector according to the invention are indicated by reference to the position of the nucleotides of the Ad5 genome as specified in the GenBank data bank, as M73260. Preference is given to an adenoviral vector according to the invention derived from the genome of a human adenovirus type 5 by deletion of: (i) the whole of the promoter controlling expression of the early proteins of the E1A region, and/or (ii) the whole of the promoter controlling the expression of the early proteins of the E1B region, and/or both of these regions.

Deletions of the E1A promoter preferably range from nucleotides 431 to 555, or more preferably ranging from nucleotides 460 to 522 and mutations of one or more nucleotides 199 to 210, or 297 to 301. Alternatively, disruption of the TATA box, cap site of the E1A promoter and insertion of a promoter of a tumor associated antigen to regulate E1A would yield a similar specificity. In some instances, disruption of the CAAT box (nucleotides 427 to 430) may also partially cripple the E1A promoter.

E1B modifications include deletion of nucleotides 1650 to 1710, or modifications in the TATA box and cap site of the E1B region wherein the modifications decrease the activity of the E1B promoter. Minimal deletion or mutation of nucleotides 1670 to 1676 and/or nucleotides 1699 to 1795 would decrease activity of E1B promoter.

In order to regulate an antisense mRNA for the E1A region by a promoter of a gene expressed by normal cells, but not tumor cells, the promoter of the normal gene can be inserted in the antisense orientation between nucleotides 1650 and 1710 or at any insertion site therein.

The TATA box and cap site in the E4 region are located at nucleotides 35630 through 35639 and 36605 through 36611, respectively. Disruption of these sequences or deletion of nucleotides 35543 to 35661, and insertion of the tumor specific promoter or tissue specific promoter would alter the regulation of the E4 genes.

According to one embodiment, an adenoviral vector according to the invention is derived from the genome of an adenovirus by deletion of the whole of the adenoviral E3 region as well as modifications described for efficient regulation of E1 region(s) by a tumor or tissue specific promoter(s). It will then be possible to insert up to 4kb of exogenous nucleotide sequence.

A preferred adenoviral vector according to the invention is derived from a human adenovirus type 5 by crippling the E1A promoter concurrent with its regulation by a tumor specific promoter, with or without modifications extending from nucleotides 460 to 522, but which may extend from nucleotides 380 to 455.

In the context of the present invention, the exogenous or heterologous gene of interest being delivered to the cancer or tumor by the vector can code either for an antisense RNA, or for a mRNA which will then be translated into a protein of interest. A gene of interest can be of genomic type, of complementary DNA (cDNA) type or of mixed type (minigene, in which at least one intron is deleted). It can code for a mature protein, a precursor of a mature protein, in particular a precursor intended to be secreted and accordingly comprising a signal peptide, a chimeric protein originating from the fusion of sequences of diverse origins, or a mutant of a natural protein displaying improved or modified biological properties. Such a mutant may be

obtained by mutation, deletion, substitution and/or addition of one or more nucleotide(s) of the gene coding for the natural protein.

Genes of interest, which are useful in the context of the present invention, include, but are not limited to: genes encoding cytokines, such as GM-CSF, interferon gamma, interleukins; genes encoding anti-angiogenic proteins such as endostatin and angiostatin, or a cellular gene, the expression of which is deregulated, for example an oncogene; genes encoding antigenic epitopes in order to increase the host cell's immunity; genes coding for major histocompatibility complex classes I and II proteins, as well as the genes coding for the proteins which are inducers of these genes; genes coding for cellular enzymes or those produced by pathogenic organisms; and suicide genes, such as the TK-HSV-1 suicide gene. For example, after administration of the corresponding prodrug, the suicide gene converts a prodrug to a toxic metabolite and the ganciclovir, a nucleotide analogue can be incorporated in DNA molecules undergoing synthesis, thereby entering the DNA of cells during replication. This incorporation enables dividing cells, such as cancer cells, to be selectively destroyed.

The invention also relates to an adenoviral particle, as well as to a eukaryotic host cell comprising an adenoviral vector according to the invention. Said cell is advantageously a mammalian cell, and preferably a human cell, and can comprise said vector in integrated form in the genome, or preferably in non-integrated (episome) form.

The subject of the invention is also the therapeutic or prophylactic use of an adenoviral vector, an adenovirus particle, a eukaryotic host cell or a replication selective adenoviral construct according to the invention.

In addition, the present invention relates to a pharmaceutical composition comprising as therapeutic or prophylactic agent an adenoviral vector, an adenovirus particle, or a replication selective vector or particle according to the invention, in combination with a vehicle which is acceptable for pharmaceutical purposes.

The composition according to the invention is intended especially for the preventive or curative treatment of disorders, such as: hyperproliferative disorders including, but not limited to, primary pulmonary hypertension, cervical hyperplasia and restenosis; and cancers, such as those induced by carcinogens, viruses and / or

dysregulation of oncogene expression. The treatment of cancer (before or after the appearance of significant symptoms) is particularly preferred.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15%, preferably by at least 50%, more preferably by at least 90%, and most preferably complete remission of a hyperproliferative disease or cancer of the host. Alternatively, a "therapeutically effective amount" is sufficient to cause an improvement in a clinically significant condition in the host. In the context of the present invention, a therapeutically effective amount of replication selective adenovirus is a number that is effective to treat a proliferative disease or tumor in a patient or host. Preferably, administration or expression of an "effective" amount (or number) of replication selective adenovirus resolves the underlying infection or cancer.

A pharmaceutical composition according to the invention may be manufactured in a conventional manner. In particular, a therapeutically effective amount of a therapeutic or prophylactic agent is combined with a vehicle such as a diluent. A composition according to the invention may be administered to a patient (human or animal) by aerosol or via any conventional route in use in the field of the art, especially via the oral, subcutaneous, intramuscular, intravenous, intraperitoneal, intrapulmonary, intratumoral, intratracheal route or a combination of routes. The administration may take place in a single dose or a dose repeated one or more times after a certain time interval. The viruses may also be administered using a producer cell technology (Coukos *et al.*, *Clin. Cancer Res.* 5:1523-1537 (1999a); Herrlinger *et al.*, *Mol. Therapy* 1:347-357 (2000)), utilizing cells that target the replication selective adenovirus to the relevant tissue and amplify the virus.

The appropriate administration route and dosage vary in accordance with various parameters, for example with the individual being treated or the disorder to be treated, or alternatively with the gene(s) of interest to be transferred. The particular formulation employed will be selected according to conventional knowledge depending on the properties of the tumor, or hyperproliferative target tissue and the desired site of action to ensure optimal activity of the active ingredients, *i.e.*, the extent to which the replication selective adenovirus reaches its target tissue or a biological fluid from which the drug has access to its site of action. In addition, these

viruses may be delivered using any vehicles useful for administration of adenovirus, which would be known to those skilled in the art. It can be packaged into capsules, tablets, etc. using formulations known to those skilled in the art of pharmaceutical formulation.

Dosages for a given host can be determined using conventional considerations, *e.g.*, by customary comparison of the differential activities of the subject preparations and a known appropriate, conventional pharmacological protocol. Generally, a pharmaceutical composition according to the invention comprises a dose of adenovirus according to the invention of between 10^4 and 10^{14}, advantageously 10^5 and 10^{13}, and preferably 10^6 and 10^{11}.

A pharmaceutical composition, especially one used for prophylactic purposes, can comprise, in addition, a pharmaceutically acceptable adjuvant, carrier, fillers or the like. Suitable pharmaceutically acceptable carriers are well known in the art. Examples of typical carriers include saline, buffered saline and other salts, liposomes, and surfactants. The adenovirus may also be lyophilized and administered in the forms of a powder. Taking appropriate precautions not to kill the replication-deficient adenovirus, the preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, and the like that do not deleteriously react with the active virus. They also can be combined where desired with other biologically active agents, *e.g.*, antisense DNA or mRNA.

The compositions and methods described herein can be useful for preventing or treating cancers of a number of types, including but not limited to breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, pancreatic cancer, gastric cancer, cervical cancer, ovarian cancer, brain cancers, various leukemias and lymphomas. One would expect that any other human tumor cell, regardless of expression of functional p53, would be subject to treatment or prevention by the methods of the present invention. The invention also encompasses a method of treatment, according to which a therapeutically effective amount of an adenoviral vector, an adenoviral particle, a eukaryotic cell or a replication selective adenovirus according to the invention is administered to a patient requiring such treatment.

Also useful in conjunction with the methods provided in the present invention would be chemotherapy, phototherapy, anti-angiogenic or irradiation therapies, separately or combined, which maybe used before or during the enhanced treatments of the present invention, but will be most effectively used after the cells have been sensitized by the present methods. As used herein, the phrase "chemotherapeutic agent" means any chemical agent or drug used in chemotherapy treatment, which selectively affects tumor cells, including but not limited to such agents as adriamycin, amsacrine, etoposide, actinomycin D, VP 16, camptothecin, colchicine, taxol, cisplatinum, vincristine, vinblastine, methotrexate. Other such agents are well known in the art.

As described above, the agents encompassed by this invention are not limited to working by any one mechanism, and may for example be effective by direct poisoning, apoptosis or other mechanisms of cell death or killing, tumor inactivation, or other mechanisms known or unknown. The means for contacting tumor cells with these agents and for administering a chemotherapeutic agent to a subject are well known and readily available to those of skill in the art.

As also used herein, the term "irradiation" or "irradiating" is intended in its broadest sense to include any treatment of a tumor cell or subject by photons, electrons, neutrons or other ionizing radiations. These radiations include, but are not limited to, X-rays, gamma-radiation, or heavy ion particles, such as alpha or beta particles. Moreover, the irradiation may be radioactive, as is commonly used in cancer treatment and can include interstitial irradiation. The means for irradiating tumor cells and a subject are well known and readily available to those of skill in the art.

The viruses of the present invention can also be used to express immunostimulatory proteins that can increase the potential anti-tumor immune response, suicide genes, anti-angiogenic proteins, and/or other proteins that augment the efficacy of these replication selective adenoviruses.

In addition, cells may be screened for their ability to support viral replication of a vector according to the present invention following their generation by contacting a layer of uninfected cells with virus particles, followed by incubation of the cells under conditions for optimal viral growth. The formation of viral plaques, or cell-free

areas in the cell layer, is the result of cell lysis caused by the expression of certain viral products. Generalized CPE or screening for expression of viral antigens also may be utilized.

Examples

The present invention is further described in the following examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. The various scenarios are relevant for many practical situations, and are intended to be merely exemplary to those skilled in the art. These examples are not to be construed as limiting the scope of the appended claims. Thus, the invention should in no way be construed as being limited to the following example, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

The constructions described below are carried out according to the general techniques of genetic engineering and molecular cloning detailed in, *e.g.*, Maniatis *et al.*, (Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, N.Y. (1989)). The steps of PCR amplification follow known protocols, as described in, *e.g.*, PCR Protocols-A Guide to Methods and Applications (ed., Innis, Gelfand, Sninsky and White, Academic Press Inc. (1990)). Notably, the oligonucleotides used to modify the Ad genome may use different restriction enzyme sites than those identified below, and may use a slightly different insertion site in the genome, without affecting the outcome of the invention. Such variations, so long as not substantial, are within the understanding of one of ordinary skill in the art.

Moreover, cells are transfected according to standard techniques, well known to a person skilled in the art. Protocols enabling a nucleic acid to be introduced into a cell may employ known methods, *e.g.*, calcium phosphate transfection (Maniatis *et al.*, 1989), DEAE-dextran techniques, electroporation, methods based on osmotic shocks, micro-injection of the selected cell, or methods based on the use of liposomes. The fragments inserted into the constructs described below are indicated according to their position in the nucleotide sequence of the Ad5 genome, as disclosed in the GenBank data bank, reference M73260.

Example 1 – A prototype replication sensitive Ad, Ad460CEA, having improved specificity for CEA positive cells.

A prototype replication sensitive adenovirus of the present invention was generated by (i) engineering the recombinant adenovirus plasmid, pAd460 (see below), (ii) inserting an promoter of a tumor-associated antigen (oncolytic promoter), *e.g.*, from carcinoembryonic antigen (CEA), so that the oncolytic promoter replaces nucleotides 460-522 of Ad5, yielding the plasmid pAd460CEA, and (iii) generating a recombinant adenovirus by homologous recombination in 293 cells (ATCC, Rockville, MD).

A. Construction of the promoter plasmid: The initial plasmid, pXC.1, which was used to generate the Ad460CEA recombinant adenovirus, was purchased from Microbix (Toronto, Ontario, Canada) and then modified as follows: using PCR techniques, a PacI site was inserted into pXC.1 to replace nucleotides 460-522. Two fragments were generated by PCR. The first fragment extended from the EcoRI site to nucleotide 460 and was generated by a two step PCR process using the oligonucleotides, CTTCAAGAATTCTCATGTTTGACAGCTTA (SEQ ID No:1) and CTCGAGTTAATTAACGTCAGCTGACTATAATAATAAAACGCC.(SEQ ID No:2).

Two step PCR was performed in 100 µl, containing 1 µg phosphorated oligonucleotide of each type, 1 x Taq buffer, 5 U Taq polymerase as follows: 5 minutes at 95°C, 3 minutes at 72°C, 25 cycles of 30 seconds at 95°C, 3 minutes at 72°C, hold at 4°C for less than 1 hour. The second fragment extended from nucleotide 523 to the Xba I site, and was generated by two step PCR using oligonucleotides, GTCGACTTAATTAATTTTCTCCTCCGAGCCGCTCCGAC (SEQ ID No:3) and GCATTCTCTAGACACAGGTGATGTCG (SEQ ID No:4).

The fragments were cloned into the TA-cloning vector using a bidirectional cloning kit (Invitrogen) (San Diego, CA). Plasmids were screened by restriction enzyme digestion with EcoRI and PacI, or with XbaI and PacI, respectively. The two fragments were excised by digesting with EcoRI/ PacI or XbaI /PacI digests, respectively, purified, quantified and ligated at a 1:1:1 molar ratio into the large EcoRI –XbaI fragment of pXC.1 to yield the plasmid pXC460.5.

B. Isolation of the CEA promoter: The CEA promoter was cloned using PCR from genomic DNA isolated from A549 (ATCC, Rockville, MD), using standard methods known in the art. The oligonucleotides used were: AGATCTTTAATTTCGAGTCATCCC (SEQ ID No:5) and AGATCTTTAATTAACTCGAGATCATCCCACCTT (SEQ ID No:6). PCR was performed as a two step procedure, as described above in step A.

C. Isolation of the recombinant replication selective Adenovirus: Two Ad5 plasmids were constructed, Ad460CEA and Ad522CEA, comprising an CEA promoter. Virus was generated by homologous recombination in 293 cells, as previously described, using the large right end fragment of a recombinant adenovirus, containing an expression cassette comprising: a Rous Sarcoma Virus promoter (RSV), a green fluorescent protein gene and a SV40 poly A site (RSV promoter, GFR-SV40 polyA) replacing the E1 region, and a blue fluorescent protein gene in the E3 region. The cassette, termed Ad5.E3BFP, as shown in Figure 2B, was constructed by the Institute for Human Gene Therapy at the University of Pennsylvania. In the resulting virus construct, pAd460CEA, the CEA promoter replaced adenovirus nucleotides 460 to 522, upstream of the E1A genes, and the blue fluorescent gene was present in the E3 region (Figure 2B). In contrast, Ad522CEA had the CEA promoter inserted at nucleotide 522, and the blue fluorescent gene was present in the E3 region (Figure 2B).

Figure 2C depicts the linearized plasmid and large right end fragment of AdGFP-E3BFP. After co-transfection and homologous recombination in 293 cells, the recombinant viruses, Ad460CEA and Ad522CEA were isolated, and plaque purified three times.

D. *In vitro* efficacy: To test for *in vitro* efficacy, two colorectal carcinoma cell lines that differ in their expression levels of CEA were tested for their sensitivity to lysis and cytopathic effect by the following four viruses:

1) A replication defective, E1 region deleted Ad5 virus, Add*l*312; (Jones *et al.*, *Cell* 17:683-689 (1979);
2) Wildtype Adenovirus 5 (wtAd5);
3) Ad460CEA; and
4) Ad522CEA.

Colo320 and LoVo cells (ATCC) are colorectal carcinoma cell lines that differ significantly in their susceptibility to cytopathic effects induced by infection with wildtype adenovirus 5. Colo320 does not express any CEA (0 antigen), whereas LoVo expresses very high levels of CEA (approximately 902 ng/10^6 cells CEA antigen).

Colo320 (40,000) and LoVo cells (20,000) were plated into 96 well plates and incubated overnight at 37°C, 5% CO_2 in humidified air. Cells were infected with each of the viruses in replicates of 6, at a multiplicity of infection (MOI) ranging 0.001 to 100 in log intervals. Cells were photographed with a camera at 20x magnification using phase contrast.

Figure 3 shows cytopathic effects on Colo320, infected with one of the four viruses at a MOI of 100 (Figures 3A, 3B, 3C, 3D), and on LoVo cells, infected with the viruses at a MOI of 0.001 after 4 days (Figures 3E, 3F, 3G, 3H). As shown in Figures 3A and 3E, infection of either Colo320 or LoVo with the replication defective adenovirus, Ad*dl*312, did not affect the growth of either cell line, and a nearly confluent monolayer, similar to that of uninfected cells, was observed (Figures 3A, 3E). In contrast, both cell lines were susceptible to infection with wildtype adenovirus (Figures 3B, 3F).

The CEA positive cell line, LoVo, was also equally susceptible to cytopathic effects and lysis by Ad522CEA and Ad460CEA (Figures 3G, 3H). Ad522CEA induced less cytopathic effects on Colo320 than wtAd5 (Figure 3C), suggesting that the CEA promoter in Ad522CEA conferred some specificity for CEA positive cells. Furthermore, Ad460CEA induced less cytopathic effects on CEA negative Colo320 cells than either Ad522CEA or wtAd5 (Figure 3D). Thus, crippling the activity of the E1A promoter, as well as inserting a tumor associated promoter, improved the specificity of the replication selective adenovirus for CEA positive colorectal cells.

E. *In vivo* efficacy: The in *vivo* efficacy of these viruses was compared in xenogeneic tumor models in nude mice. Two tumor cell lines, A549 (CEA positive) and HeLa, (ATCC) were used a representative models to establish cancerous tumors in the animal models. The two were selected because they differ significantly in their expression of the CEA antigen. A549 cells are from a non small cell lung carcinoma

that expresses significant levels of CEA antigen, while HeLa cells are from a cervical cancer that typically expresses about 20-fold less CEA than A549 cells.

Cell lysates were prepared as previously described (Molnar-Kimber *et al*, *Hum. Gene Ther.* 9:2121-2133 (1998). Fifty micrograms of each cell lysate were electrophoresed into 10% polyacrylamide gel, and the proteins were transferred to nitrocellulose overnight using a dri-blot apparatus (Fisher Scientific). CEA protein was detected by immunoblotting with rabbit anti-human CEA antibody (1:100, Accurate Biochemicals), as described (Molnar-Kimber *et al*, *Hum. Gene Ther.* 9:2121-33 (1998)). Lane 1: Hela cells. Lane 2: MCF7 cells. Lane 3: A549 cells. As shown by Western blot, when tested, the expression level of CEA was at least 10-fold lower in HeLa cells than in A549 cells, which is consistent with previously reported findings.

Both of these cell lines are similarly susceptible to wildtype Ad5. A549 cells (4×10^4) or HeLa cells (4×10^4), respectively in 200 µl media, were inoculated subcutaneously into each flank, respectively, of groups of 12 week old female NCR/NCI immunocompromised (nude mice) (Taconic, Germantown, NY). The growth of the tumor nodules was monitored by periodic measurement with calipers. When most tumors were 70 - 100 mm^3, the mice were separated into groups (only tumors in this size range were used in the experiment). Tumors were injected (intratumoral injection) with.(i) 100 µl control media alone (RPMI 1640); (ii) 100 µl containing 10^9 plaque forming units (pfu) of wild type Ad5 (wtAd5), or Ad5 containing TK in the E3 region (Ad.E3tk), (iii) 100 µl containing 10^9 pfu of AdCEA460, or (iv) 100 µl containing 10^9 pfu of AdCEA522.

The growth of the tumors was monitored and the volumes were calculated. After approximately 14 days, mice ($n \geq 7$/group) were sacrificed, tumors were excised and weighed. Each experiment was performed at least 2 times and the mean $\pm$ S.E. is shown, $^*p \leq 0.05$, $^{**}p \leq 0.01$.

Measurements indicated that WtAd5 and Ad522CEA treatments significantly reduced both tumor growth and tumor weight of both A549 and HeLa tumors in comparison to treatment with media alone (Figure 5A,B). Similarly, AdCEA460 reduced A549 tumor weight by 47 $\pm$ 11% ($p<0.01$) (Figure 5A) in comparison to media control treatment group. In contrast, Ad460CEA did not significantly decrease

the tumor burden (weight) of the HeLa tumors which express no or very low levels of CEA, (−10±16% not significant) (Figure 5B) Taken together, these data indicate that deletion of the Ad5 sequences between nt 460 and 522, *i.e.*, elimination of several transcriptional regulatory elements, improved the specificity of the resulting replication selective Ad, Ad460CEA for CEA positive cells.

Example 2 – Mutations in the Enhancer.

In the alternative, mutations in the enhancer type I of E1A promoter may be accomplished using the oligonucleotides:

GCGCCGGTGTACACAGG ACCGGT CAATTTTCGCGCGGTTTTAGG (SEQ ID No: 7); and

CGCGGGAAAACTGAATAAAAGCTTGTGAAATCTGAATAATTTTGTGTTACTCGG (SEQ ID No.: 8) using the Site mutagenesis Transformation Kit (Clontech).

These modifications are incorporated into pAd460CEA. Recombinant replication selective adenoviruses are generated by homologous recombination in 293 cells. The resultant virus, Ad460eh1CEA are useful for treating cancers positive for CEA antigen expression. A promoter of a tumor associated antigen or tissue specific gene can be substituted for CEA to yield replication selective adenoviruses with the desired specificity.

Example 3 Mutations in the E1B Sequence.

Insertion of the sites for regulation of the E1B region are accomplished using the oligonucleotides: GCAGGCGTGTTAAATGGGG CTCGAGATATCACGCGT CTCATGGAGGCTTGGG (SEQ ID No: 9); and

GGGCTAATCTTGGTTAC CTCGAGATATCACGCGT ATCTGACCTCATGGAGGC (SEQ ID No: 10) using the Site mutagenesis Transformation Kit (Clontech).

A promoter from a tumor associated antigen, such as the catalytic subunit of telomerase, is inserted in the restriction sites generated by the modification is incorporated into the adenoviral plasmid, pAd460CEA to generate the plasmid, pAd460CEABTERT. Recombinant adenoviruses are generated by homologous recombination in 293 cells or other appropriate cells. The resultant virus, Ad460CEABTERT are useful for treating cancers positive for CEA antigen

expression and TERT expression. A promoter of a tumor associated antigen or tissue specific gene can be substituted for CEA promoter or the TERT promoter to yield replication selective adenoviruses with the desired specificity.

Example 4 – Antisense Adenovirus constructs.

Genes that are expressed in normal tissue, as compared with tumor tissue, are dependent on the tissue of origin of the tumor and include, in at least some tumors, but are not limited to, E-cadherin (Imao *et al., J. Urol.* 161:692-698 (1999), Mialhe *et al., Anal. Cell Pathol* 13:125-136 (1997)), uroplakin (Finch *et al., Int. J. Cancer* 80:533-538 (1999)), or others (Jerome and Muller, *Hum. Gene Ther.* 9: 2653-2659 (1998)). A promoter of one of the aforementioned genes or another gene that is not expressed in the tumors of interest are inserted between the E1A and the E1B promoters in an antisense orientation (between nucleotides 1650 and 1710) in pAd460CEA or Ad460, with a promoter from a different tumor associated antigen.

Recombinant adenoviruses are generated by homologous recombination in 293 cells or other appropriate cells. The resultant virus, Ad460CEA-ASnor are useful for treating cancers positive for CEA antigen expression. A promoter of a tumor associated antigen or tissue specific gene can be substituted for CEA promoter or the promoter of the gene expressed in normal but not tumor cells to yield replication selective adenoviruses with the desired specificity.

In sum, replication selective adenoviruses using the promoter of carcinoembyronic antigen (CEA) have been engineered and evaluated *in vivo* against two tumors induced by A549 and HeLa cells. As shown in Figure 5A and 5B, Adenovirus Ad460CEA significantly decreased the growth of A549 tumors as well as wildtype adenovirus, but did not decrease the growth of HeLa cells (low CEA expression). In contrast the virus, which only inserted the CEA promoter in between the E1A promoter and the translational site of the E1A proteins, Ad522CEA, significantly decreased tumor growth of both CEA positive A549 and HeLa cells.

Each and every patent, patent application and publication that is cited in the foregoing specification is herein incorporated by reference in its entirety.

While the foregoing specification has been described with regard to certain preferred embodiments, and many details have been set forth for the purpose of

illustration, it will be apparent to those skilled in the art that the invention may be subject to various modifications and additional embodiments, and that certain of the details described herein can be varied considerably without departing from the spirit and scope of the invention. Such modifications, equivalent variations and additional embodiments are also intended to fall within the scope of the appended claims.

What is claimed is:

1. A replication selective adenovirus mutant under the control of a tumor or tissue specific promoter, wherein said adenovirus selectively replicates at a level at least ten-fold greater within a cancer or hyperproliferative cell, as compared with replication in a normal cell, permitting the adenovirus to spread throughout a tumor nodule or hyperproliferative cells, and wherein at least adenovirus E1A promoter has been deactivated or crippled, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type adenovirus.

2. The adenovirus of claim 1, wherein adenovirus E1B or E4 promoters are also deactivated or crippled.

3. The adenovirus of claim 1, wherein said tumor specific promoter is selected from tumor types that express a tumor associated antigen.

4. The adenovirus of claim 3, having improved selectively for tumors or hyperproliferative cells expressing said tumor associated antigen.

5. The adenovirus of claim 4, wherein said tumor associated antigen is selected from the group consisting of carcinoembryonic antigen, probasin, prostate specific antigen, prostate specific membrane antigen, erb1 or erb2, α-fetoprotein, Egr-1, L-plastin, gonatotropin hormone, glycoprotein hormone α subunit, hexokinase II, glial fibrillary acidic protein, neuronal-specific enolase, MUC-1 ("DF-3"), tyrosinase, ERBB2, survivin, catalytic subunit of telomerase, IGFII P3 and P4, H19, cell cycle associated proteins, E2F-1, c-myb and the tissue specific promoter, thyroglobulin.

6. The adenovirus of claim 5, wherein said tumor associated antigen is carcinoembryonic antigen.

7. The adenovirus of claim 3, wherein there is improved selectivity *in vivo* for any target cell which expresses the adenovirus vector under the control of the tumor specific promoter.

8. The adenovirus of claim 3, which has improved selectivity for tumors or hyperproliferative cells selected from the group consisting of cancers, carcinomas, sarcomas or neoplasms of the prostate, lung, gastrointestine, colorectum, pancreas, breast, ovary, cervix, stomach, thyroid, mesothelioma, liver, kidney, skin, head and neck, brain, as well as leukemias and lymphomas.

9. The adenovirus of claim 8, wherein there is improved selectivity for non-small cell lung carcinoma.

10. A method of delivering to a target cell a heterologous gene or gene fragment encoding a therapeutic peptide or polypeptide, by delivering the adenovirus vector of claim 1, wherein said vector is under the control of a tumor specific promoter, and wherein at least adenovirus E1A promoter is disabled or crippled.

11. A method of delivering to a target cell a suicide gene or therapeutic gene by delivering the adenovirus vector of claim 1, wherein said vector is under the control of a tumor specific promoter, and wherein at least adenovirus E1A promoter is disabled or crippled.

12. A method of introducing an adenovirus of claim 1 to a target cell by delivering said adenovirus vector to the target cell, wherein said vector is under the control of a tumor specific promoter, and wherein at least adenovirus E1A promoter is disabled or crippled.

13. The target cell comprising the adenovirus of claim 1.

14. A method of treating a patient suffering from a cancer, carcinoma, sarcoma, neoplasm, leukemia, lymphoma or hyperproliferative cell disease by

administering to said patient a therapeutically effective amount of a replication selective adenovirus mutant under the control of a tumor specific promoter, wherein said adenovirus selectively replicates at a level at least ten-fold greater within said cancer, sarcoma, neoplasm, leukemia, lymphoma or hyperproliferative cell, as compared with replication in a normal cell, permitting the adenovirus to spread throughout a tumor nodule or hyperproliferative cells, and wherein at least adenovirus E1A promoter has been deactivated or crippled, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type adenovirus.

15. The method of treatment of claim 14, wherein adenovirus E1B or E4 promoters are also deactivated or crippled.

16. The method of treatment of claim 14, wherein said tumor specific promoter is selected from tumor types that express a tumor associated antigen.

17. The method of treatment of claim 16, having improved selectively for tumors or hyperproliferative cells expressing said tumor associated antigen.

18. The method of treatment of claim 17, wherein said tumor associated antigen is selected from the group consisting of carcinoembryonic antigen, probasin, prostate specific antigen; prostate specific membrane antigen, erb1 or erb2, α-fetoprotein, Egr-1, L-plastin, gonatotropin hormone, glycoprotein hormone α subunit, hexokinase II, glial fibrillary acidic protein, neuronal-specific enolase, MUC-1 ("DF-3"), tyrosinase, ERBB2, survivin, catalytic subunit of telomerase, IGFII P3 and P4, H19, cell cycle associated proteins, E2F-1, c-myb and the tissue specific promoter, thyroglobulin.

19. The method of treatment of claim 18, wherein said tumor associated antigen is carcinoembryonic antigen.

20. The method of treatment of claim 17, wherein there is improved selectivity *in vivo* for any target cell which expresses the adenovirus vector under the control of the tumor specific promoter.

21. The method of treatment of claim 17, which has improved selectivity for tumors or hyperproliferative cells selected from the group consisting of cancers, carcinomas, sarcomas, lymphomas, or neoplasms of the prostate, lung, gastrointestine, colorectum, pancreas, breast, ovary, cervix, thyroid, gastric, renal, mesothelioma, head and neck, and brain.

22. The method of treatment of claim 21, wherein there is improved selectivity for non-small cell lung carcinoma.

23. The method of treatment of claim 14, wherein the hyperproliferative cell disease is selected from the group consisting of restenosis, intimal proliferative disease and primary pulmonary hypertension.

24. The method of treatment of claim 14, wherein the therapeutically effective amount of the replication selective adenovirus mutant delivers to a target cell a heterologous gene or gene fragment encoding a therapeutic peptide or polypeptide.

25. The method of treatment of claim 24, wherein more of a heterologous gene or gene fragment encoding a therapeutic peptide or polypeptide, is delivered to the target cell than was previously possible, thereby achieving a direct, oncolytic effect on the target.

26. The method of treatment of claim 14, wherein the therapeutically effective amount of the replication selective adenovirus mutant delivers to a target cell a suicide gene or therapeutic composition.

27. A method for producing an infectious, replication selective adenovirus particle comprising:

(a) selecting a tumor specific promoter selected from a tumor that express a tumor associated antigen, which is active in a eukaryotic cell;

(b) deactivating or crippling at least adenovirus E1A promoter in a replication selective adenovirus, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type replication selective adenovirus;

(c) introducing the promoter of step (a) with the crippled replication selective adenovirus of step (b), thereby placing the replication selective adenovirus under the control of the tumor associated antigen promoter;

(d) culturing said adenovirus construct under conditions permitting the uptake of said adenovirus vector by, and replication in, a host cell expressing the tumor associated antigen; and

(e) harvesting said infectious, replication selective adenovirus particle produced by said host cells, wherein the resulting adenovirus particle is selectably reproduced only in cells expressing the tumor associated antigen.

28. The adenovirus particle produced by the method of claim 27.

29. The method of claim 27, wherein said adenovirus vector further comprises introducing a heterologous gene or gene fragment encoding a therapeutic peptide or polypeptide, wherein said gene or gene fragment is selected from the group consisting of genes coding for oncogenes, tumor suppressor genes, antisense and ribozyme RNAs, genes encoding enzymes, genes encoding cytokines and other immune modulating macromolecules, genes encoding recombinant antibodies, genes encoding lytic peptides, genes encoding vaccine antigens, genes encoding macromolecules which complement genetic defects in somatic cells, and genes encoding macromolecules which catalyze processes leading to cell death.

30. A method of inactivating a tumor or hyperproliferative target cell in a patient, comprising the steps of:

(a) selecting a tumor specific promoter selected from a tumor that express a tumor associated antigen, which is active in a eukaryotic cell;

(b) deactivating or crippling at least adenovirus E1A promoter in a replication selective adenovirus, thereby reducing activity of the promoter to a significantly lower level than that of the wild-type replication selective adenovirus;

(c) introducing the promoter of step (a) with the crippled replication selective adenovirus of step (b), thereby placing the replication selective adenovirus under the control of the tumor associated antigen promoter to provide a vector;

(d) introducing into the vector of step (c) a heterologous gene or gene fragment encoding therapeutic peptide or polypeptide, such that it will be expressed from the vector within the target cell;

(e) introducing the vector of step (d) into the target cell of the patient in a therapeutically effective amount.

31. The method of claim 30, wherein the target cell is further treated by chemotherapy, immunostimulation, phototherapy, irradiation, or any combination thereof.

32. A pharmaceutical composition comprising the replication selective adenovirus particle of claim of claim 28, and a pharmaceutically acceptable carrier therefor, wherein said pharmaceutical composition, when administered in a therapeutically effective amount to a cancer, carcinoma, sarcoma, neoplasm, leukemia, lymphoma or hyperproliferative target cell of a patient, achieves an oncolytic effect on the target.

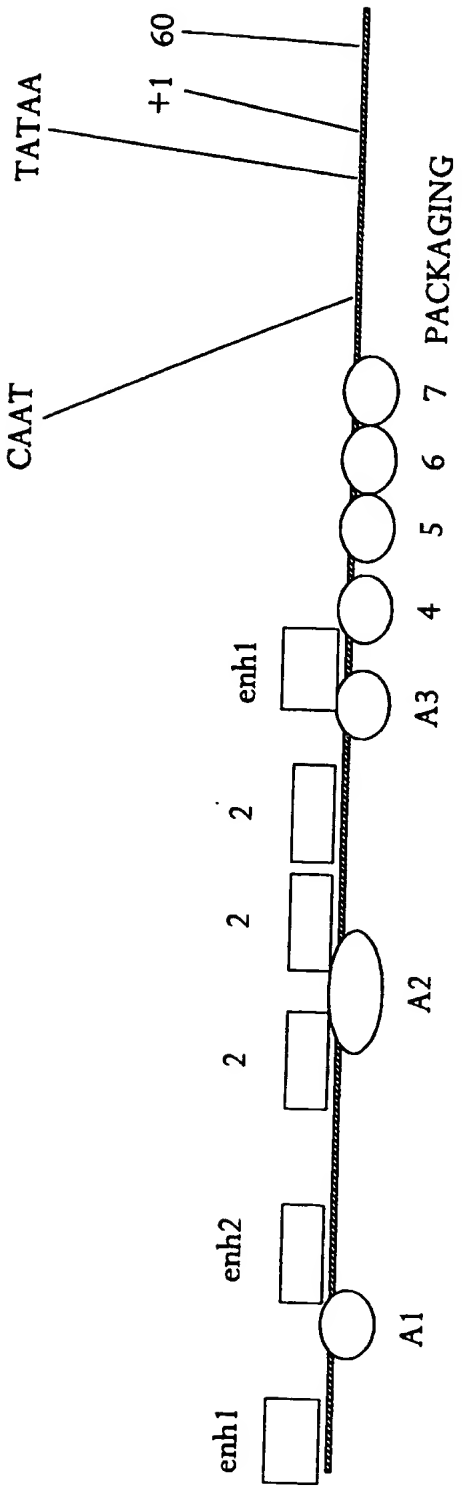

FIG. 1

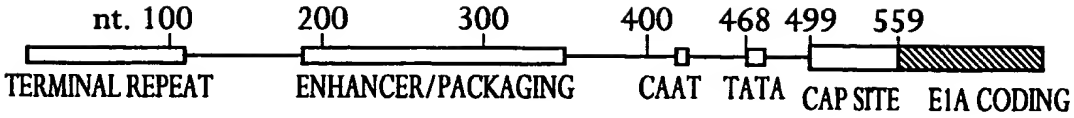
nt. 100
200
300
400
468
499
559
TERMINAL REPEAT
ENHANCER/PACKAGING
CAAT
TATA
CAP SITE
E1A CODING

FIG. 2A

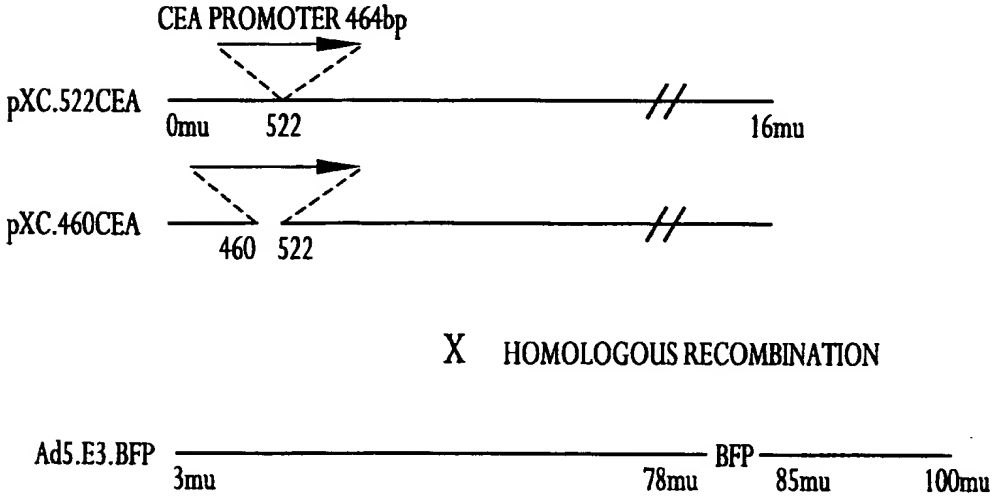
CEA PROMOTER 464bp
pXC.522CEA
0mu
522
16mu
pXC.460CEA
460
522
X HOMOLOGOUS RECOMBINATION
Ad5.E3.BFP
3mu
78mu
BFP
85mu
100mu

FIG. 2B

Colo320 LoVo

Add/312

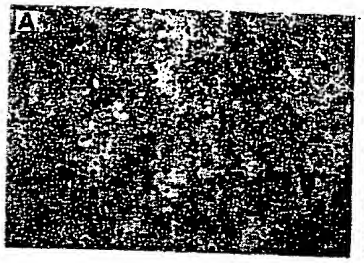

FIG. 3A

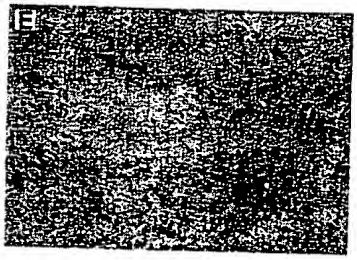

FIG. 3E

Wt Ad

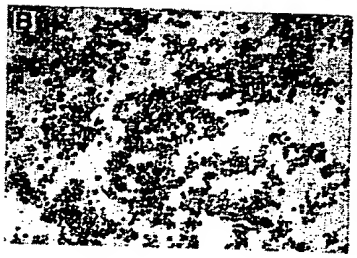

FIG. 3B

FIG. 3F

Ad522CEA

FIG. 3C

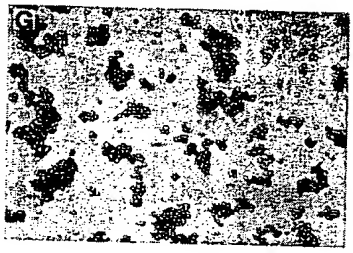

FIG. 3G

Ad460CEA

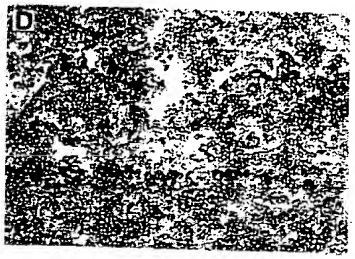

FIG. 3D

FIG. 3H

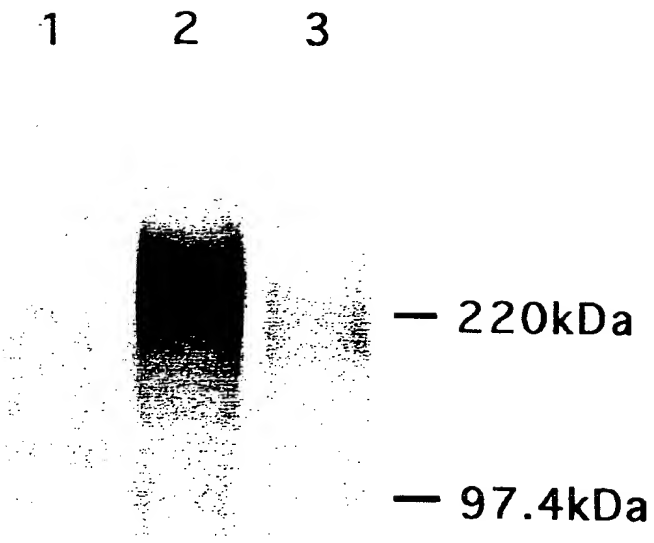

FIG. 4

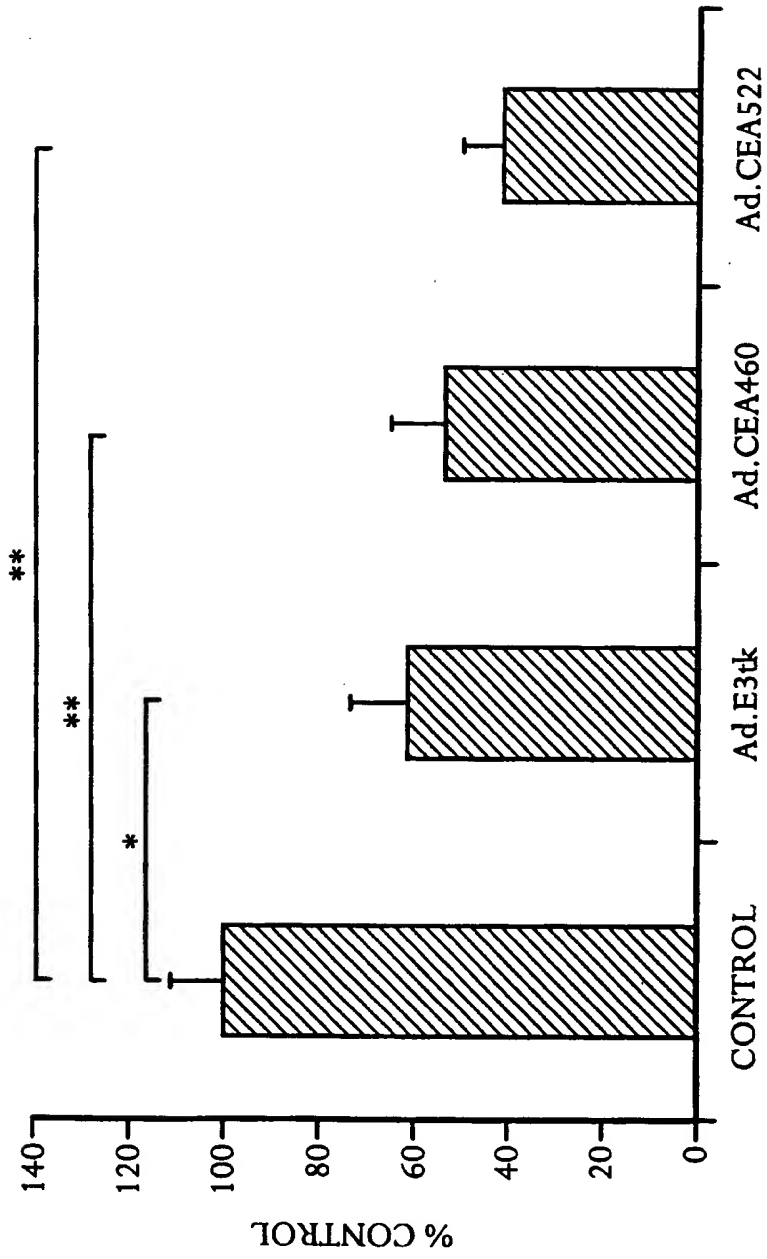
% CONTROL
140
120
100
80
60
40
20
0
CONTROL
Ad.E3tk
Ad.CEA460
Ad.CEA522
ADENOVIRUS
*
**
**

FIG. 5A

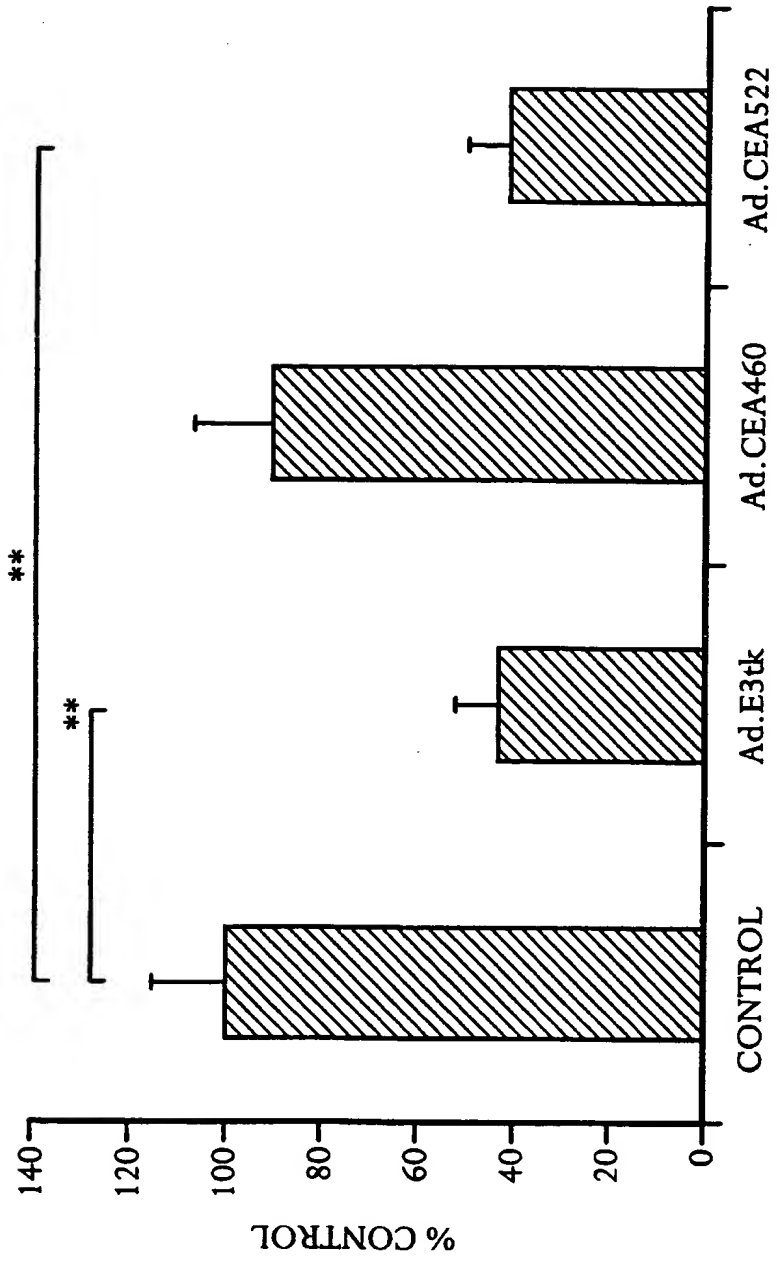
% CONTROL
140
120
100
80
60
40
20
0
**
**
CONTROL
Ad.E3tk
Ad.CEA460
Ad.CEA522
ADENOVIRUS

FIG. 5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27212

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 48/00; A01N 63/00; C12Q 1/68; C12N 5/00, 15/63

US CL :424/93.2, 93.6; 435/6, 325, 455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 93.6; 435/6, 325, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST, CAPLUS, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96/17053 A1 (GENETIC THERAPY, INC.) 06 June 1996, complete document, EspeciallyE page 17 para 1, page 17 para 3 lines 7-10, page 19 para 3 lines 2-3, page 22 para 2, page 29 para 1 line 9 and para3, page 30 para 1.	1-32
X	WO 96/34969 A2 (CANJI, INC.) 07 November 1996, complete document, especially page 8 line 4-17; page 11 lines 8-15; page 18 para 1.	1,3-5,7,8, 10-14,16-18, 20-21, 23-26,30, 32
.... Y		..... 2,15, 27-29

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 12 DECEMBER 2000	Date of mailing of the international search report 16 JAN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BHARATI R. DHRUVA PH.D. Telephone No. (703) 308-0196

TERRY J. DEY PARALEGAL SPECIALIST TECHNOLOGY CENTER 1

Form PCT/ISA/210 (second sheet) (July 1998) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/27212

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZIEGER M.A. et al. Adenoviral Infection of Thyroid Cells: A Rational for Gene Therapy for Metastatic Thyoid Carcinoma. Surgery. 1996, Vol. 120, No. 6, pages 921-925, see page 921 line 8-11 and para 4 line 1-3.	5
X	RODRIGUEZ R. et al. Prostate Attenuated Replication Competent Adenovirus(ARCA) CN706: A selective Cytotoxic for Prostate-specific Antigen-positive Prostate Cancer Cells. Cancer Research 1997. Vol. 57, No. 5, pages 2559-2563, complete article.	1,3,4,5,7, 8,12,13,14, 16,17,18, 20,21,
Y	MAUCERI H.J. et al. Tumor Necrosis Factor alpha (NF-alpha) Gene Therapy Targeted by Ionizing Radiation Selctively Damages Tumor Vasculature. Cancer Research. 1996, Vol. 56, pages 4311-4314, see especially page 4311 para2 lines 8-12 and 16-20.	1-5, 31
Y	KATABI M.M. et al Hexokinase Type II: A Novel Tumor Specific Promoter for Gene Targeted Therapy Diffentially Expressed and Regulated in Human Cancr Cell. Human Gene Therapy. 20 January 1999, Vol. 10, pages 155-164, especially page 156 para 5 line 3-6.	1-5 and 9
Y	MORELLI A.E. et al. Neuronal and Glial Cell Type Specific Promoters within Adenovirus Recombinants Restric the Expression of the Apoptosis-inducing Molecule Fas Ligand to Predetermined Brain Cell types, and Abolish Peripheral Liver Toxicity. Journal of Ggeneral Virology. 1999, Vol. 80, pages 571-583, especially page 572, para 7 lines 22-47.	1-5